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Mass spectrometry-based characterization of the vitreous phosphoproteome

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Abstract

Purpose—The vitreous gel is a highly hydrated extracellular matrix containing many proteins. These proteins are likely accumulated in the vitreous by local secretion, filtration from the blood, or diffusion from the surrounding tissues and vasculature, and may be altered in disease state. In the last several years, several reports of large-scale profiling of vitreous proteins have been published; however, there is little information on the characterization of the phosphoproteome of vitreous. Here, we sought to identify phosphopeptides and their phosphorylation sites from vitreous.

Experimental design—We used titanium dioxide (TiO₂) to enrich phosphopeptides from vitreous and identified them by liquid chromatography coupled tandem mass spectrometry (LC-MS/MS). Results: We identified 85 unique phosphopeptides and the phosphorylation sites from 44 proteins.

Conclusions and clinical relevance—We present a method for characterization of phosphoproteome from vitreous samples using current mass spectrometry technologies and yielded an initial assessment of the phosphoprotein/peptide content of human vitreous, thus providing important biological information toward further understanding of the post-translational modifications of vitreous proteins and their functional significance in disease.

Keywords

Titanium dioxide; phosphopeptide; vitreous; crystallin; LTQ-Orbitrap; mass spectrometry

1. Introduction

The vitreous gel is a highly hydrated extracellular matrix whose structure is maintained by a network of collagen fibrils and filled with glycosaminoglycan hyaluronan. It has been demonstrated that the vitreous has certain physiological functions, such as providing a reservoir of nutrients for the metabolic requirements of the lens, excluding cells and large macromolecules from the vitreous cavity in order to maintain transparency [1], coordinating eye growth [2,3], and protecting the eye during mechanical trauma [4,5]. The proteins in the vitreous are likely accumulated there by local secretion, filtration from the blood, or diffusion from the surrounding tissues and vasculature [6,7]. Recently, several lines of

evidence revealed that the proteins retained in the closed vitreous chamber may be altered in disease state: (1) significant changes in human vitreous humour were observed following cataract extraction [8]; (2) higher abundance of pigment epithelium-derived factor, a potent inhibitor of angiogenesis, was detected in vitreous with diabetes [9]; (3) elevated levels of vascular endothelial growth factor (VEGF) was found in vitreous from patients with diabetic retinopathy and retinal-vein occlusion [10]; (4) increased amount of extracellular carbonic anhydrase-I was characterized in vitreous from individuals with diabetic retinopathy [11]; (5) levels of phosphorylated VEGF receptor in the vitreous of eyes with wet age-related macular degeneration was noted to correlate with response to anti-VEGF therapy [12].

Despite the sophisticated imaging technology and treatment modalities available to ophthalmologists, many eye diseases cannot be diagnosed until they are at a stage where irreversible, vision-limiting changes have occurred. Ocular diseases are increasing in prevalence. In part this is because many ocular diseases arise as a consequence of the normal aging process, with many retinal disorders caused by vascular changes associated with diabetes as well as sclerotic processes. Thus, there is a tremendous unmet need for developing new diagnostic technologies to identify protein molecules or their modified forms in the vitreous that could be early warning for pathologies or markers of a particular disease stage that cause severe vision loss. In the last several years, proteomics studies by mass spectrometry technique identified more than 500 proteins in vitreous, including multiple post-translationally modified forms of metabolic, antioxidant, angiogenic, and immune modulatory proteins [13–18], however, there is little information on the characterization of the phosphoproteome of vitreous. Post-translational phosphorylation is a common and important mechanism of acute and reversible regulation of protein function in mammalian cells. Dynamic phosphorylation of proteins on serine, threonine and tyrosine residues is recognized as a key mode of regulating cell cycle, cell growth, cell differentiation and metabolism [19,20]. In this present work, we report an approach to identify a large number of phosphopeptides and their phosphorylation sites using titanium dioxide (TiO₂)-based phosphopeptide enrichment followed by liquid chromatography coupled nano-electrospray tandem mass spectrometry (LC-MS/MS) analysis. The result yielded an initial assessment of the phosphoprotein/peptide content of human vitreous, which can serve as a launch-point for further exploration and analysis.

2. Materials and methods

2.1. Materials

Dithiothreitol (DTT), iodoacetamide, urea, ammonium bicarbonate, 2,5-dihydroxybenzoic acid (DHB), trifluoroacetic acid (TFA), and phosphoprotein bovine beta-casein were from Sigma-Aldrich; Coomassie (Bradford) protein assay kit was from Pierce; trypsin was from Promega; Human angiotensin I (AngI) peptide DRVYIHPFHL, and human tyrosine phosphorylated Angiotensin II (AngII-P) DRVpYIHPF were from Calbiochem; phosphopeptide DRFP SVNLDVK was synthesized from Peptide 2.0 Inc.

The clinical vitreous samples were obtained from 11 patients undergoing total vitrectomy under IRB approval (Istituto Superiore di Sanit, Italy) and patient consent (Table 1). Immediately after collection, samples were diluted with Lactated Ringer's solution (the solution is isotonic with blood and intended for intravenous administration, containing 130 mM sodium ion, 109 mM chloride ion, 28 mM lactate, 4 mM potassium ion, and 1.5 mM calcium ion from sodium chloride, sodium lactate, calcium chloride and potassium chloride), and centrifuged at 5000 g for 7 minutes at 4 °C to eliminate insoluble material. The supernatant was transferred to another tube, and the protein concentration was measured by Bradford Assay (BioRad). The samples were stored at –80 °C until use.

2.3. Trypsin digestion and desalting

Proteins from 1 mg vitreous, pooled from several patients, were spiked with the phosphoprotein bovine beta-casein as an internal standard, reduced by 10 mM DTT in the presence of 8 M urea for 30 minutes at 37 °C, and then alkylated by 50 mM iodoacetamide at room temperature. The concentrated urea in the sample was diluted to a final concentration of 2 M, and the proteins were digested by trypsin at 37 °C for 6 hours in a buffer containing ammonium bicarbonate (50 mM, pH 9). The digestion mixture was then acidified by adding glacial acetic acid to a final concentration of 2% and desalted by SepPak C₁₈ column (Waters, catalogue number WAT054955).

2.3. TiO₂ enrichment of phosphopeptides

Phosphopeptides were enriched from the mixture of all tryptic peptides using TiO₂ column (200 µm × 2 cm) packed in-house [21]. 1 pmol of standard phosphopeptide angiotensin II phosphate was added to the SepPak-cleaned sample. The sample then was mixed with an equal volume of Loading Buffer (200 mg/mL DHB, 5% TFA, 80% acetonitrile), and loaded into the TiO₂ column using the Pressure Cell (Brechtbühler Inc.) with flow rate of 3 µL per minute. The column was washed by 200 µL of Wash Buffer 1 (40 mg/mL DHB, 2% TFA, 80% acetonitrile) and 2 × 200 µL of a second Wash Buffer 2 (2% TFA, 50% acetonitrile) to remove non-phosphopeptides. Phosphopeptides were eluted from the column with the Elution Buffer (5% ammonia solution). Ammonia in the eluate was removed by lyophilization (~3 minutes), and the sample was acidified by adding glacial acetic acid to a final concentration of 2%, and desalted by ZipTip (Millipore, catalogue number ZTC18S960). 100fmol of standard peptide angiotensin I were spiked into the enriched sample as an internal standard.

2.4. Mass spectrometry for phosphopeptide identification

The purified phosphopeptides were analyzed by reversed-phase liquid chromatography coupled nanospray tandem mass spectrometry (LC-MS/MS) using an LTQ-Orbitrap mass spectrometer (Thermo Fisher) [21]. The reversed-phase LC column was slurry-packed in-house with 5 µm, 200 Å pore size C₁₈ resin (Michrom BioResources, CA) in a 100 µm i.d. × 10 cm long piece of fused silica capillary (Polymicro Technologies, Phoenix, AZ) with a laser-pulled tip. After sample injection, the column was washed for 5 minutes with mobile phase A (0.1% formic acid), and peptides were eluted using a linear gradient of 0% mobile phase B (0.1% formic acid, 80% acetonitrile) to 45% mobile phase B in 120 minutes at 200 nL/minute, then to 100% B in an additional 5 minutes. The LTQ-Orbitrap mass spectrometer was operated in a data-dependent mode in which each full MS scan (60,000 resolving power) was followed by eight MS/MS scans where the eight most abundant molecular ions were dynamically selected and fragmented by collision-induced dissociation (CID) using a normalized collision energy of 35%. The “FT master scan preview mode”, “Charge state screening”, “Monoisotopic precursor selection”, and “Charge state rejection” were enabled so that only the 2+ and 3+ ions were selected and fragmented by CID. Tandem mass spectra were searched against the NCBI human protein database using SEQUEST (Bioworks 3.3.1, ThermoFisher) with full tryptic cleavage constraints, static cysteine alkylation by iodoacetamide, and variable phosphorylation of Ser/Thr/Tyr and methionine oxidation. Mass tolerance for precursor ions was 10 ppm and mass tolerance for fragment ions was 0.25 Da. Confident phosphopeptide identifications were determined using stringent filter criteria for database match scoring followed by manual evaluation of the results.

3. Results

The phosphopeptides from trypsinized vitreous (~ 1 mg) were enriched by TiO₂ column and analyzed by LC-MS/MS using LTQ-Orbitrap mass spectrometer. The SEQUEST search

results were filtered using the following criteria: “ranked top #1; Xcorr versus charge 1.8, 2.5 for 2+, 3+ ions; mass accuracy 3 ppm; probability of randomized identification of peptide < 0.05”, which yielded 548 matched MS2 spectra. Among these, 253 (46.2%) spectra were matched to non-phosphopeptides, and 295 (53.8%) spectra were matched to phosphopeptides. A total of 85 unique phosphopeptides was identified from 44 proteins (Table 2). The estimated false discovery rate (FDR) was lower than 1% by searching a combined forward-reversed database as described by Elias [22]. Example CID spectra from three of the identified phosphopeptides, LVGGPMDApSVEEEGVRR from cystatin C, GLMMELpSEDCPSIQDR from crystallin, gamma C, and KANDEpSNEHSDVIDSQELSK from secreted phosphoprotein 1 isoform b, are shown in Figures 1. In addition to the matched b-ions and y-ions, the obtained CID spectra exhibited neutral loss of phosphate group from parent ions, which enabled the confident identification of these phosphopeptides. To confirm the validity of the spectral findings, one of these identified phosphopeptides, DRFPpSVNLDVK from crystallin, alpha B, was obtained as synthetic peptides, and its MS2 spectrum was acquired using the LTQ-Orbitrap. The MS2 spectrum of this phosphopeptide obtained from TiO₂ enriched vitreous was almost identical to the MS2 spectrum of synthesized peptide (Figures 2), indicating accurate identification of this phosphopeptide.

As shown in Table 2, some of these identified phosphopeptides were from abundant serum proteins such as albumin, apolipoproteins, kininogen 1, serine (or cysteine) proteinase inhibitor, secreted phosphoprotein 1, and alpha-2-HS-glycoprotein. These proteins have also been shown to be phosphorylated in our previous characterization of serum phosphoproteome [21]. In addition, several cellular phosphopeptides such as ITHSpPTVSQVTER from ankyrin 1 isoform 1, LSpSSWESLQPEPSHPY from spectrin beta isoform b, HIQETEWQpSQEGK from SPARC-like 1 protein, were identified as well. Interestingly, 9 phosphopeptides from three types of crystallins—i.e., LPpSNVDQSALSCSLSadGMLTFCGPK from alpha A, RPFpFHPpSPSR, APpSWFDpTGLSEMR, and DRFPpSVNLDVK from alpha B, MEFTpSSCPNVsERSFDNVR and MEFpTSSCPNVsERSFDNVR from beta A3, VLpSGAWVGFEHAGFQGQQYILER from beta A4, SIIVSAGPWVAFEQpSNFR from beta B1, and GLMMELpSEDCPSIQDR from gamma C, were also identified from vitreous. It is well-known that crystallins are the principal components of eye lens, contributing about 20–60% of total wet weight [23].

We next examined the phosphopeptides from patients with different diseases. For this purpose, vitreous from patient 2 and 4 who had retinal detachment without diabetes were pooled to generate Group 1, and vitreous from patient 6 and 10 who had hemovitreous with diabetes were pooled to generate Group 2. Using similar approach, phosphopeptides were enriched from two groups of trypsinized vitreous by TiO₂ column, analyzed by LC-MS/MS, and compared with each other. As a result, phosphopeptides from abundant serum proteins such as albumin, apolipoproteins, kininogen 1, and secreted phosphoprotein 1, were identified from both groups, whereas the 9 phosphopeptides from three types of crystallins were found only in Group 2, indicating that the phosphorylation of crystallins were involved in the disease of hemovitreous with diabetes.

Discussions

Protein phosphorylation can affect catalytic activity, localization of a protein in the cell, protein stability, and the ability of a protein to dimerize or form a stable complex with other molecules. Protein phosphorylation and dephosphorylation function together in signal transduction pathways to induce rapid changes in response to hormones, growth factors, and neurotransmitters [19,20]. To understand exactly why a particular protein becomes phosphorylated, it may be necessary to identify precisely which amino acid residues are

phosphorylated. These residues then can be changed by site-directed mutagenesis, and the mutated protein can be examined for changes in activity, intracellular localization, and association with other proteins in the cell. In addition, identification of phosphorylation sites could reveal which protein kinase regulates the protein and thereby help to understand the biological function and significance of signal transduction pathway. Today, mass spectrometry (MS)-based methods are the most commonly used technique for global profiling of phosphoproteins and phosphorylation sites [24]. However, due to the low stoichiometry of most phosphorylation, enrichment of phosphopeptides by immobilized metal ion affinity chromatography (IMAC), or titanium dioxide (TiO₂) chromatography, is advantageous or required before MS analysis [25].

While there have been reports that bovine crystallin alpha B was a phosphoprotein [26–28], and phosphorylation of rat alpha B was involved in uveitis progression and inflammatory responses [29], the large scale identification of phosphoproteins and mapping their phosphorylation sites in vitreous, to our knowledge, has never been reported. The vitreous proteome, containing more than 500 proteins based on recent proteomics studies [13–18], is a very complex matrix predominated by high-abundance resident proteins, such as albumin and apolipoproteins, together with proteins that are shed or secreted by key ocular structures. The complexity of vitreous protein mixture and the high background level of the most abundant proteins make MS-based phosphoprotein identification and localization of phosphorylation sites a challenge. Here, in order to obtain an improved characterization of vitreous phosphoproteome, we conducted the first attempt and successfully identified a large number of phosphopeptides and their phosphorylation sites using TiO₂-based phosphopeptide enrichment followed by LC-MS/MS analysis using an LTQ-Orbitrap instrument which combines high sensitivity, high resolution and high mass accuracy.

In this study, phosphopeptides from trypsinized clinical vitreous samples (~ 1mg), pooled from several patients, were enriched by TiO₂ column and analyzed by LTQ-Orbitrap mass spectrometer. Due to a) the high concentrations of resident proteins such as albumin and apolipoproteins in vitreous, b) the low abundance stoichiometry of phosphorylated proteins in the biological samples, c) the lack of sufficient volume and amount of clinical vitreous samples, we chose to analyze the pooled vitreous (~ 1 mg) instead of vitreous from a single patient in order to yield the highest number of identified phosphopeptides and provide the most comprehensive view of the vitreous phosphoproteome. This effort was not meant to serve as a complete description of the vitreous phosphoproteome nor identify disease-specific biomarker candidates, but as an initial effort to begin the description of this potentially important information archive.

In addition to the phosphopeptides from cellular proteins and abundant serum proteins, we identified several phosphopeptides from all three types of crystallins in this pilot study. Among these, Ser19 of phosphopeptide RPFPPFHpSPSR and Ser59 of phosphopeptide APpSWFDTGLSEMR from crystalline alpha B are the homologous residues of phosphorylated rat alpha B that was involved in uveitis progression and inflammatory responses [29]. As mentioned previously, the vitreous proteins, shed or secreted by surrounding ocular structures such as the retina and the lens, provide a record of the health or disease state of the ocular tissue. We therefore compared the identified phosphopeptides from patients who had retinal detachment without diabetes and patients who had hemovitreous with diabetes, and found that phosphopeptides from crystallins were identified only in patients who had hemovitreous with diabetes. The preliminary findings from this comparison, as usual, require further study to determine their relevant role in the disease.

It is not clear what kinds of kinases are responsible for the phosphorylation of these proteins and what is the function of these vitreous phosphoproteins, however, these modified forms

of proteins are definitely a reflection of ongoing physiological and pathological events, and could be early warnings for diseases or stages of diseases such as diabetic retinopathy or macular degeneration. Further investigation of these phosphoproteins may lead to a better understanding of their roles in both normal and disease conditions. We believe that vitreous phosphoproteome may be a future source of candidate biomarkers for individualized therapy of age-related macular degeneration and other ocular diseases.

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Figure 1.

CID spectra of three phosphopeptides. CID spectra were labeled to show singly-charged b and y ions, as well as ions corresponding to neutral losses of the phosphate group and water. A) phosphopeptide LVGGPMDApSVEEEGVRR from cystatin C; B) phosphopeptide GLMMELpSEDCPSIQDR from crystallin, gamma C; C) phosphopeptide KANDEpSNEHSDVIDSQELSK from secreted phosphoprotein 1 isoform b.

Table 1

The vitreous samples used for the study.

Patient ID	Protein concentration (mg/mL)	Eye disease	Age	Gender
1	3.70	hemovitreous, no diabetes	59	female
2	3.04	retinal detachment, no diabetes	74	male
3	1.57	hemovitreous, no diabetes	73	female
4	5.23	retinal detachment, no diabetes	78	female
5	4.69	retinal detachment, no diabetes	70	female
6	12.71	diabetes with hemovitreous	69	female
7	3.41	diabetes with hemovitreous	69	female
8	1.73	diabetes with ischemic cardiopathy	59	male
9	9.29	diabetes with hemovitreous	77	female
10	17.67	diabetes with hemovitreous	82	female
11	4.97	diabetes with retinopathy	59	male

Table 2The identified vitreous phosphopeptides by LTQ-Orbitrap from TiO₂ enrichment.

Protein description	Position ^a	Phosphopeptides sequence
crystallin, alpha A	120–145	LPpSNVDQSALSCSLSadGMLTFCGPK
crystallin, alpha B	12–22	RPFFPFHpSPSR
	57–69	APpSWFDTGLSEMR
	73–82	DRFpSVNLDVK
crystallin, beta A3	46–64	MEFTpSSCPNVSErSFDNVR
	46–64	MEFpTSSCPNVSErSFDNVR
crystallin, beta A4	49–71	VLpSGAWVGFEHAGFQGQQYILER
crystallin, beta B1	93–110	SIIVSAGPWVAFEQpSNFR
crystallin, gamma C	100–115	GLMMELpSEDCPSIQDR
albumin precursor	76–88	TCVADepSAENCdK
transferrin	385–399	IECVpSAETTEDCIAK
	684–698	CSTSSLLEACpTFRRP
alpha 1 globin	129–140	FLApSVSTVLTSK
	129–140	FLASVpSTVLTSK
beta globin	42–60	FFEpSFGDLSTPDaVMGNPK
	42–60	FFESFGDLSpTPDaVMGNPK
delta globin	42–60	FFESFGDLSpSPDaVMGNPK
	68–83	VLGAfpSDGLAHLdNLK
secreted phosphoprotein 1 isoform a	52–70	QNLLAPQNAVpSpSEETNDFK
secreted phosphoprotein 1 isoform b	21–35	QADSGSpSEEKQLYNK
	190–206	AIPVAQDLNAPSDWDpSR
	190–206	AIPVAQDLNAPpSDWDSR
	190–206	AIPVAQDLNAPpSDWDpSR
	207–227	GKDSYETSQLDDQpSAETHSHK
	207–227	GKDpSYETSQLDDQpSAETHSHK
	235–254	KANDEpSNEHSDVIDSQELSK
	235–254	KANDESNEHSDVIDpSQELSK
	235–254	KANDESNEHpSDVIDpSQELSK
	235–254	KANDESNEHSDVIDSQELSKVpSR
	235–254	KANDEpSNEHSDVIDSQELpSKVpSR
	286–300	FRISHELDSApSSEVN
	286–300	FRIpSHELDSASSEVN
	286–300	FRIpSHELDpSASSEVN
	286–300	FRIpSHELDpSApSSEVN
secreted phosphoprotein 1 isoform c	31–50	QNAVpSSEETNDFKQETLPSK
	31–50	QNAVpSpSEETNDFKQETLPSK
chromogranin A precursor	319–331	GGKpSGELEQEEER
chromogranin B precursor	134–153	ADEPQWSLYPSDSQVpSEEVK
	179–202	GEDpSSEEKHLPEPGETQNAFLNER

Protein description	Position ^a	Phosphopeptides sequence
	255–277	GQPRpSQEEpSEEGEEDATSEVDK
	259–277	SQEEpSEEGEEDATSEVDKR
	372–387	APRPQpSEESWDEEDKR
	397–409	MAHGYGEEpSEER
transmembrane protein 132A isoform a	515–536	VPGAEGPAEPAEApSDEAERR
glycophorin A precursor	120–150	KSPSDVKPLPSPDTPVPLSSVEIENPETpSDQ
inter-alpha globulin inhibitor H2 polypeptide	55–76	SLPGEpSEEMMEEVDQVTLYSYK
myocilin	194–216	AVPPGpSREVSTWNLDTLAFQELK
clusterin isoform 1	438–460	VTTVASHTSDpSDVPSGVTEVVVK
serine (or cysteine) proteinase inhibitor, clade C (antithrombin), member 1	62–78	ATEDEGpSEQKIPEATNR
kininogen 1	325–343	ETpTCSKESNEELTESCETK
	325–343	ETTCSEpSNEELTESCETK
	325–343	ETTCpSEpSNEELTESCETK
	416–427	AGAEPApSEREVS
SPARC-like 1	71–89	SSVLKpSKEEpSHEQSAEQGK
	187–220	DQGNQEQDPNIpSNGEEEEKEPGEVGTHTNDNQR
	227–255	EHANpSKQEEDNTQSDILEESDQPTQVSK
	256–286	MQEDEFDQGNQEQEDNpSNAEMEEENASNVNK
	287–299	HIQETEWQpSQEGK
	409–426	KAENSpSNEETSSEGNMR
alpha-2-HS-glycoprotein	132–143	CDSSDPpSAEDVR
	318–337	HTFMGVVSLGSPpSGEVSHPR
apolipoprotein A-II preproprotein	52–62	VKpSPELQAEAK
apolipoprotein E precursor	138–152	GEVQAMLGQpSTEELR
apolipoprotein L1 isoform a precursor	306–320	VTEPISAEpSGEQVER
	306–320	VTEPIpSAESGEQVER
	306–320	VTEPIpSAEpSGEQVER
secretory granule, neuroendocrine protein 1 (7B2 protein)	199–211	SVPHFpSDEDKDPE
fibrinogen, alpha polypeptide isoform alpha-E preproprotein	354–367	PGSTGTWNPGpSSER
sodium-phosphate cotransporter IIa C-terminal-associated protein 2	19–46	FNPKLGIDNPVLpSLAEDHDPpYDPWPpSLER
chondroitin sulfate proteoglycan 2 (versican)	2108–2124	QEIESETTpSEEQIQEEK
ankyrin 1 isoform 1	777–787	LGYIpSVTDVLK
	804–828	MSFPETVDEILDVpSEDEGEELISFK
	1683–1695	ITHSPpTVSQVTER
	1683–1695	ITHpSPTVSQVTER
spectrin beta isoform b	2122–2137	LSpSSWESLQPEPSHPY
	2122–2137	LSpSpSWESLQPEPSHPY
cystatin C precursor	35–51	LVGGPMDApSVEEEGVRR
ATP-binding cassette, sub-family B, member	6744–763	APGIILLDEATpSALDTSNER
frizzled-related protein	295–313	HLGLSKpSDSSNSDSTQSQK
amyloid precursor-like protein 1 isoform 1 precursor	348–369	QALNEHFQSIQLTLEEQVpSGER

Protein description	Position ^a	Phosphopeptides sequence
PREDICTED: similar to retinitis pigmentosa 1-like 1	169–183	PDAGSEEPDpSEGGR
tenascin XB isoform 1	3678–3702	LGPLSAEGTTGLAPAGQTSEEpSRPR
nuclear ubiquitous casein kinase and cyclin-dependent kinase substrate	9–28	KVVDYSQFQEpSDDADEDYGR
vitronectin precursor	311–324	DpSWEDIFELLFWGR
glyceraldehyde-3-phosphate dehydrogenase	163–186	VIHDNFGIVEGLMTTVHAITApTQK

^aPosition of the initial and final peptide amino acids in the protein sequence.