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(54) **Artificial protein, method for absolute quantification of proteins and uses thereof**

Künstliches Protein, Verfahren für absolute Quantifizierung der Proteine und seine Benutzung

Protéine artificielle, méthode pour la quantification absolue des protéines et son utilisation

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(73) Proprietor: **Polyquant GmbH**
93051 Regensburg (DE)

(72) Inventors:
• **Pratt, Julie M.**
Wigston,
Leicester LE 18 1NP (GB)
• **Beynon, Robert**
Prenton,
Merseyside CH43 7PU (GB)
• **Gaskell, Simon**
Altrincham,
Cheshire WA14 9NJ (GB)

(74) Representative: **Elbel, Michaela**
Rothkopf Theobald Elbel
Patentanwälte, Partnerschaft
Isartorplatz 5
80331 München (DE)

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Description

Field of the invention

[0001]   The present invention relates to proteomics and more specifically to absolute quantification of proteins.

Background of the invention

[0002]   The need for absolute quantification in proteomics is becoming increasingly urgent. The most promising method is based on stable isotope dilution involving simultaneous determination of representative proteolytic peptides and stable isotope labeled analogs. The principal limitation to widespread implementation of this approach is the availability of standard signature peptides in accurately known amounts.

[0003]   The two primary themes in proteomics are protein identification and the comparison of protein expression levels in two physiological or pathological states (comparative proteomics). The long term goal of being able to define the entire proteome of a cell is still unrealized, but the characterization of many thousands of proteins in a single analysis is now attainable.

[0004]   For proteomics to become a platform technology serving the emergent field of systems biology, there is a pressing need for enhancement of quantification (Righetti, Eur J Mass Spectrom 10 (2004), 335-348). Most comparative proteomics studies deliver *relative* quantification, expressing the changes in amount of a protein in the context of a second cellular state (for example Dunkley, Mol Cell Proteomics (2004); Hoang, J Biomol Tech 14 (2003), 216-233; Ong, Mol Cell Proteomics (2002), 376-386).

[0005]   However, the goal must ultimately be to define the cellular concentrations of proteins absolutely, whether as molarities or as numbers of molecules per cell. Absolute quantification, which poses one of the greatest challenges in proteomics, draws on well-established precepts in analytical chemistry, and requires either external standards or internal standards (Sechi, Curr Opin Chem Biol 7 (2003), 70-77; Julka, J Proteome Res 3 (2004), 350-363). External standard-ization is typified by immunodetection, whether solution phase or on position-addressable antibody arrays (Walter, Trends Mol Med 8 (2002), 250-253; Lopez, J Chromatogr B Analyt Technol Biomed Life Sci 787 (2003), 19-27). The second approach, reliant on internal standardization, is based on mass spectrometry (MS), wherein highly selective detection of ions (or ion fragmentations) characteristic of the analytes of interest is combined with the use of internal standards.

[0006]   In the most rigorous MS analyses, stable isotopic variants of the analytes are used as internal standards. The key underlying principle is that the determination of *relative* signal intensities during mass spectrometric analysis can be converted into *absolute* quantities of analyte by reference to an authentic standard available in known amounts. Direct application of this approach to intact proteins is impractical, and it is common to adopt the principle of surrogacy, that is to quantify indirectly by reference to a proteolytic peptide derived from the protein of interest.

[0007]   Analyses based on these principles have been dubbed "AQUA" (absolute quantification) using internal stand-ards synthesized *de novo* by chemical methods (Gerber, PNAS 100 (2003), 6940-6945). However, this approach does not lend itself well to absolute quantification of large numbers of proteins, as each Q-peptide would need to be chemically synthesised and independently quantified.

[0008]   The international patent application PCT/US03/17686, published as WO 03/102220 provides methods to de-termine the absolute quantity of proteins present in a biological sample. The principle of WO 03/102220 is based on the generation of an ordered array of differentially isotopically tagged pairs of peptides, wherein each pair represents a unique protein, a specific protein isoform or a specifically modified form of a protein. One element of the peptide pairs is a synthetically generated, external standard and the other element of the pair is a peptide generated by enzymatic digestion of the proteins in a sample mixture. For performing the method of WO 03/102220 the standard peptides are calibrated so that absolute amounts are known and added for comparison and quantification. A sample of interest is also labelled with the same isotope tag as used for the standard peptides except differing in the isotopic label. The pairs of signals, which correspond to differentially labelled sample and standard peptides are finally observed and related to a list of expected masses based on the particular standard peptides included. The disadvantage of WO 03/102220 is that standard peptides need to be individually synthesised, purified and quantified. Moreover, both the sample and the standard peptides need to be specifically labelled separately, increasing the potential for variability between experiments.

[0009]   WO 2004/013636 discloses compounds with an alkyl epitope tag site and a protease cleavage site and a method for simultaneously identifying and determining the levels of expression of cysteine-containing proteins in normal and perturbed cells. First and second protein samples and first and second peptide samples are prepared and reacted with the disclosed compound, a proteolytic cleavage step is carried out and an affinity purification step is conducted.

[0010]   WO 03/102220 provides methods of determining the absolute quantity of proteins present in a biological sample. An ordered array of differentially isotopically tagged pairs of peptides is provided. One element of the peptide pairs is a synthetically generated, external standard and the other element of the pair is a peptide generated by enzymatic digestion

of the proteins in a sample mixture.

[0011] US 2002/0037532 discloses a method for protein identification in complex mixtures that utilises affinity selection of constituent proteolytic peptide fragments unique to a protein analyte. These peptides are also called "signature peptides" which function as analytical surrogates. With the help of mass spectrometric analysis of the proteolyzed mixture a protein in a complex sample can be identified without purifying the protein.

[0012] Thus there is still an existing need to develop easy and convenient methods for absolute quantification in proteomics.

Summary of the invention

[0013] The present invention is directed to an artificial protein generated by gene design de novo comprising concatamers of Q-peptides which are signature peptides, generated by any proteolytic or chemical fragmentation for quantitative analysis of the proteome of a sample, cell or organism, comprising:

(a) at least two consecutive peptides linked by a cleavage sequence for separating the peptides;
(b) a singular marker on one or more peptides for determination of the absolute amount of the protein; and
(c) N-terminal and C-terminal extensions for protection, purification and quantification of the peptides;

wherein each peptide represents one single protein of the sample, cell or organism, the peptide is unique within the set of Q-peptides and each peptide is in a defined stoichiometry, and wherein the protein comprises 20 - 60 peptides.

[0014] For the purpose of the invention the artificial protein is also named QCAT protein and the peptides used for the QCAT protein are called Q-peptides.

[0015] The cleavage sequence between two Q-peptides may be an enzymatic or a chemical cleavage sequence.

[0016] The N-terminal and C-terminal extensions protect the quantification peptides from processing and exoproteolysis. The artificial protein further includes features which allow for easy purification of the Q-CAT protein.

[0017] Each of the Q-peptides represents one single protein of the sample, cell or organism and each peptide is in a defined stoichiometry, which is typically, but not exclusively 1:1.

[0018] The present invention further concerns a collection of Q-peptides, which covers the complete proteome of an organism. This collection allows for rapid quantification of the proteome of such an organism.

[0019] The present invention also concerns a vector comprising the QCAT protein and a kit comprising the vector and/or the QCAT protein.

[0020] Moreover, the invention is directed to a method for quantitative analysis of the proteome of a sample, cell or organism, comprising the steps of:

(a) quantifying the absolute amount of the artificial protein of any of claims 1-10 comprising concatamers of Q-peptides and N-terminal and C-terminal extensions for protection, purification and quantification or one peptide containing the singular marker;
(b) generating a preparation of the proteins to be quantified;
(c) mixing the products of steps (a) and (b);
(d) completely cleaving the artificial protein of any of claims 1 - 10 and the proteins to be quantified in step (b) at the cleavage sequence;
(e) determining the mass of peptides, and from this;
(f) calculating the absolute amount of each protein,

wherein the artificial protein and/or the peptides are isotopically labelled.

[0021] It is important to note that the proteins do not have to be purified - partially purified followed by high resolution separation technologies would be equally acceptable.

Brief description of the drawings

[0022]

Figure 1 shows examples of Q-peptides selected as signature peptides;
Figure 2 shows the DNA sequence, translated protein sequence and features of the QCAT;
Figure 3 shows the Characterisation of the QCAT protein and Q-peptides;
Figure 4 shows the Quantification using the QCAT protein, and
Figure 5 shows the Use of the QCAT for muscle protein quantification.

Detailed description of the invention

[0023] The inventors describe here the design, expression and use of artificial proteins that are concatamers of tryptic Q-peptides for a series of proteins, generated by gene design *de novo.* The artificial protein, a concatamer of Q-peptides ("QCAT") is designed to include both N-terminal and C-terminal extensions. The function of the extensions is to protect the true Q-peptides, to introduce a purification tag (such as a His-tag) and a sole cysteine residue for quantification of the QCAT.

[0024] The novel gene is inserted into a high-level expression vector and expressed in a heterologous expression system such as *E. coli.* Within the QCAT protein, each Q-peptide is in a defined stoichiometry (typically, but not exclusively 1:1), such that the entire set of concatenated Q-peptides can be quantified in molar terms by determination of the QCAT protein. Moreover, the QCAT protein is readily produced in unlabelled or labelled form by growth of the expression strain in defined medium containing the chosen label.

[0025] The inventors have successfully designed and constructed an artificial gene encoding a concatenation of tryptic peptides (a QCAT protein) from over 20 proteins. The protein further includes features for quantification and purification. The artificial protein was expressed in *E.coli* and synthesis of the correct product was proven by mass spectrometry. The QCAT protein is readily digested with trypsin; is easily quantified and can be used for absolute quantification of proteins. This strategy brings within reach the accurate and absolute quantification of large numbers of proteins in proteomics studies. Additionally, the QCAT was labelled by selective incorporation of a stable isotope labelled amino acid or by incorporation of ^{15}N nitrogen atoms at every position in the protein.

[0026] It is therefore an object of the present invention to provide an artificial protein for quantitative analysis of the proteome of a sample, cell or organism, comprising:

(a) at least two consecutive peptides linked by a cleavage sequence for separating the peptides;
(b) a singular marker on one or more peptides for determination of the absolute amount of the protein; and
(c) N-terminal and C-terminal extensions for protection of the peptides;

wherein each peptide represents one single protein of the sample, cell or organism, the peptide is unique within the set of Q-peptides and each peptide is in a defined stoichiometry, and wherein the protein comprises 20-60 peptides.

[0027] In a preferred embodiment of the invention, the artificial protein comprises 20 - 60 peptides, most preferred 60 peptides. It is possible to include multiple instances of specific peptides to modify the stoichiometry.

[0028] In yet another embodiment, the cleavage sequence is cleaved by a protease, preferably by trypsin and the singular marker is a cysteine residue.

[0029] Further, one or more peptides of the artificial protein are repeated identically at least one time, preferably one or more times, to achieve a particular stoichiometry between all peptides sequences.

[0030] It is preferred to include an affinity tag (e.g. a histidine tag) for purification of the protein. It is particularly preferred to include the affinity tag in either the N-terminal or C-terminal extensions of the protein.

[0031] In a further embodiment, the protein is labelled by an isotope, which is selected from the group consisting of ^{13}C, ^{15}N, ^{2}H and ^{18}O.

[0032] In yet a further embodiment each peptide comprises between about 3 and 40 amino acids, preferably about 15 amino acids.

[0033] The protein may comprise a molecular weight of about 10 - 300 kDa, preferably about 150 - 200 kDa, most preferred about 150 kDa and is preferably expressed in *E. coli.*

[0034] The origin of the proteome, i.e. the sample, cell or organism is preferably a mouse, rat, ape or human, but can be from any proteinaceous source.

[0035] In a preferred embodiment of the invention, the peptides represent different conformational, metabolic or modification states of the protein, in order to quantify all proteins derived posttranslationally from such a protein.

[0036] Further, the peptides of the artificial protein have preferably a defined molecular weight distribution and quantitative ratios. A mass spectrometer can be calibrated, preferably with molecular weights, which result in equidistant mass spectroscopy signals. Preferably, one or more peptides are represented twice in order to unambiguously label a reference molecular weight for calibration.

[0037] The invention is further directed to a collection of concatamers of Q-peptides as defined in claim 1, which covers the complete proteome of an organism. All expressed proteins of an organism are defined as the proteome of such organism. This allows for rapid quantification of the proteome of such organism.

[0038] Further included in the disclosure of the present invention is the absolute quantification of the protein levels of a certain reference strain, which can then be used to compare the protein levels of this particular strain or similar strains under varying experimental conditions.

[0039] The invention is also directed to a vector comprising a nucleic acid encoding the artificial protein and a kit comprising the artificial protein and/or the nucleic acid encoding the artificial protein. Further the invention is directed to

a method for quantitative analysis of the proteome of an organism, which is described in detail above.

[0040] The present invention will be better understood by the encompassed examples and results with reference to the accompanying figures.

Detailed description of the figures

Figure 1. Examples of Q-peptides selected as signature peptides.

[0041] For a series of proteins, peptides were selected (using multiple criteria) from proteins identified as the abundant proteins in a soluble fraction of chicken skeletal muscle, and assembled into an artificial protein, or Q-cat. Left side: Coomassie blue stained, SDS-PAGE analysis of soluble chicken muscle proteins. Center: MALDI-ToF spectrum of tryptic digestion of gel slices corresponding to selected protein bands. The peptide ion labelled with an oval represents the peptide chosen for inclusion in the Q-cat protein, Right side: mass and position of the indicated signature peptides within the designed Q-cat protein. Details of the peptides are in Table 1.

Figure 2. The DNA sequence, translated protein sequence and features of the QCAT.

[0042] The DNA sequence of the synthetic gene, with relevant cloning sites, is shown on the top line and the derived amino acid sequence is shown below. The grey blocked areas indicate the extent of the tryptic peptides, with the donor chicken proteins, tryptic peptide assignment (T1-T25) and the peptide mass (in Da) indicated. A non-cleavable Arg-Pro tryptic site within phosphoglycerate kinase (boxed) is included to confirm the non-digestibility of this site. Peptides (white boxes) encode the initiator methione, N-terminal sacrificial sequence and spacer sequences, and are not derived from proteins of interest. The black boxes highlight the sequences carrying the unique cysteine residue for quantification and Hiss tag for purification. T1 and T2 are sacrificial peptides designed to protect the N-terminus of the first true Q-peptide (T3)

Figure 3. Characterisation of the QCAT protein and Q-peptides

[0043] The pET21a/QCAT plasmid was transformed into *E. coli* DE3 cells and after a period of exponential growth the expression of the QCAT was induced with IPTG. The cell lysates from pre-induced and induced cells were compared on SDS-PAGE (inset). After solubilization of the pellet, and affinity chromatography on a NiNTA column, the purified QCAT protein was homogeneous, and was digested in solution with trypsin. The peptides were analysed on MALDI-ToF mass spectrometry. The inset tryptic digestion map is shaded to indicate the relative intensities of signals corresponding to each peptide in the mass spectrum; peptides smaller than 900 Da, derived from the 'sacrificial' parts of the QCAT are less readily detected in this type of mass spectrometric analysis due to interfering ions.

Figure 4. Quantification using the QCAT protein

[0044] The QCAT protein was prepared in unlabelled form (L: "light") and in a form uniformly labelled with ^{15}N (H: "heavy"). The H and L QCAT proteins were separately purified, quantified and mixed in different ratios, before tryptic digestion and measurement of peptide intensities by MALDI-ToF mass spectrometry. Panel a) illustrates the mass spectrum for the Q-peptide for adenylate kinase (GFLIDGYPR, 12 nitrogen atoms). In panel b) the measured L:H ratios were plotted relative to the mixture ratio, in a triplicate series of experiments for which individual points are shown. In the bottom panel, the data for seven peptides are collated and expressed as mean $\pm$ SD (n=18-21). The dotted line defines the 95% confidence limits of the fitted straight line.

Figure 5. Use of the QCAT for muscle protein quantification.

[0045] A preparation of soluble proteins from skeletal muscle of chicks at 1d and 27d was mixed with [^{15}N] QCAT, digested with trypsin and analysed by MALDI-ToF MS. For a subset of proteins, it was possible to determine the intensities of the endogenous and standard peptide, and from this, calculate the absolute amounts (in nmol/g tissue) of each protein. Three animals were used at each time point, error bars are SEM (n=3). Proteins were AK: adenylate kinase, ApoA1: apoliporotein A1, LDHB: lactate dehydrogenase B, Beta Trop: beta tropomyosin, Beta Eno: beta enolase, GP: glycogen phosphorylase, ALDO B: aldolase B, TPI: triose phosphate isomerase, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, Actin, API: actin polymerization inhibitor, PK: pyruvate kinase and CK: creatine kinase.

1. Design of the gene encoding the Q-protein concatamer

[0046] One of the inventors' major interests is in proteome dynamics (Pratt, Mol Cell Proteomics 1 (2002), 579-591),

and in changes in protein expression during muscle development (Doherty, Proteomics 4 (2004), 2082-2093; Doherty, Proteomics in press (2005)). A system that shows dramatic developmental changes in protein expression is the chicken pectoralis skeletal muscle from immediately post-hatching to maturity. Accordingly, for the demonstration QCAT set, the inventors chose twenty chicken proteins that had been previously identified as changing in expression level in developing skeletal muscle (Doherty, Proteomics 4 (2004), 2082-2093). A single tryptic fragment was chosen to represent each protein (a "Q-peptide"), although a peptide that can be reproducibly generated by any proteolytic or chemical fragmentation could be used, and in this example, Q-peptide selection was based on theoretical and experimental criteria. The first criterion was that the Q-peptides should lack a cysteine residue, as cysteine residue could be used for quantification of the QCAT and the absence of cysteines should avoid complex intra- and inter-molecular disulphide bond formation in the expressed protein. Secondly, the peptide chosen should be unique within the set of Q-peptides. Thirdly, the Q-peptides were chosen with masses between 1000 Da and 2000 Da, corresponding to the region in MALDI-ToF mass spectra where sensitivity of detection is typically high and interfering signals are low. Finally, an operational criterion was added, inasmuch as the inventors selected peptides that were already demonstrated to give a strong signal on MALDI-ToF mass spectrometry 75% (15 out of 20) of which were Arg-terminated tryptic peptides - the propensity of such peptides to give stronger signals on MALDI-ToF mass spectrometry is well documented (Brancia, Electrophoresis 22 (2001), 552-559). A final, less important criterion was that the Q-peptides should contain at least one instance of an abundant and chemically refractory amino acid such as leucine or valine, as this would facilitate metabolic labeling with amino acids for the preparation of stable isotope labeled Q-peptides. The peptides are summarized in Table 1:

Table 1 - Peptides selected for Q-cat protein

	Peptide mass (Da)	Sequence	N atoms	Parent protein
T1	405.2	MAGK	5	Construct, sacrificial
T2	386.25	VIR	6	Construct, sacrificial
T3	1036.52	GFLIDGYPR	12	Adenylate kinase
T4	1601.87	WLAYEPVWAIGTGK	16	Triose phosphate isomerase
T5	1176.57	NLAPYSDELR	14	Apolipoprotein A1
T6	1193.56	GDQLFTATEGR	15	Myosin binding protein C
T7	1789.88	SYELPDGQVITIGNER	21	Alpha actin
T8	1291.67	QVVESAYEVIR	15	Lactate dehydrogenase B
T9	1390.74	LITGEQLGEIYR	16	Beta enolase
T10	1361.63	ATDAESEVASLNR	17	Alpha tropomyosin
T11	1160.58	SLEDQLSEIK	12	Myosin heavy chain (embryonic)
T12	1441.68	VLYPNDNFFEGK	15	Glycogen phosphorylase
T13	1489.71	GILAADESVGTMGNR	19	Aldolase B
T14	1345.64	ATDAEAEVASLNR	17	Beta tropomyosin
T15	1687.8	LQNEVEDLMVDVER	19	Myosin heavy chain (adult)
T16	1748.77	LVSWYDNEFGYSNR	19	Glyceraldehyde 3-phosphate dehydrogenase
T17	1767.98	ALESPERPFLAILGGAK	21	Phosphoglycerate kinase
T18	1249.65	QVVDSAYEVIK	13	Lactate dehydrogenase A
T19	1803.93	AAVPSGASTGIYEALELR	21	Alpha enolase
T20	1823.97	LLPSESALLPAPGSPYGR	21	Actin polymerization inhibitor
T21	1857.9	FGVEQNVDMVFASFIR	25	Pyruvate kinase
T22	1991.95	GTGGVDTAAVGAVFDISN ADR	25	Creatine kinase
T23	274.15	AGK	4	Construct, sacrificial
T24	892.42	VICSAEGSK	10	Construct, quantification

(continued)

	Peptide mass (Da)	Sequence	N atoms	Parent protein
T25	1408.68	LAAALEHHHHHH	24	Construct, purification tag

[0047] Once the candidate set was nominated, the Q-peptides were assembled and a gene was constructed, which encoded the assembled Q-peptides using codons for maximal expression in *E. coli.* At the C-terminus an extension was added to provide a cysteine residue and a His tag purification motif (the latter provided in this case by the vector pET21a). An additional series of amino acids was appended to the N-terminus to provide an initiator methionine residue and a sacrificial peptide, which when cleaved would expose a true Q-peptide (Figure 1). This avoided complications due to N-formylation or removal of methionine from the N-terminus of QCAT. The transcript encoded by the initial QCAT gene was then analyzed *in silico* for features such as hairpin loops that might compromise translation. If such a feature was noted, the order of the Q-peptides was swapped until an acceptable mRNA structure was obtained - the sequence of Q-peptides within a QCAT is not relevant to their use as quantification standards and the order is thus amenable to such manipulation. The gene was constructed from a series of overlapping oligonucleotides and confirmed by DNA sequencing.

2. Expression of the QCAT

[0048] The QCAT gene was constructed with restriction sites, such that it could be inserted into a range of expression vectors (Figure 2). In this instance, the gene (confirmed by DNA sequencing) was inserted into pET21 a at the Ndel and *Hind*III sites and was expressed initially in *E.coli* (NovaBlue (DE3)) grown in rich medium. After induction by IPTG, SDS-PAGE analysis confirmed high-level expression of a protein of the expected mass (~35 kDa). This protein was present in the insoluble fraction of sonicated cells, and was presumed to be the QCAT protein present in inclusion bodies. From this preparation we purified the QCAT protein by affinity chromatography using Ni-NTA resin, which resulted in a homogeneous preparation (Figure 2, inset). The intact average mass of this protein, measured by ESI-MS was 33036 ± 2 Da (data not shown), compared to the predicted mass of 33167 Da for the QCAT protein, a difference of 131 Da which is exactly consistent with loss of the methionine residue from the N-terminus. The approx. 35 kDa gel band was subjected to in-gel digestion with trypsin and analysed by MALDI-ToF MS. All predicted QCAT peptides were readily observed in the MALDI-ToF mass spectrum, although the N and C-terminal sacrificial peptidic material yielded, by design, fragments that were too small to be seen in the MALDI-ToF mass spectrum (Figure 3). Although the peptides were chosen to yield good signals on MALDI-ToF, some peptides were markedly less intense than others. These included all of the lysine-terminated peptides, with the exception of T17, which included a non-cleavable Arg-Pro site, which although lysine terminated still yielded a strong signal on MALDI-ToF mass spectrometry. This was particularly evident in the isoform-specific Q-peptides T8 and T18, derived from lactate dehydrogenases A and B (QVVESAYEVIR and QVVDSAYEVIK respectively) - the lysine terminated peptide was less than 10 % of the intensity of the arginine-terminated peptide, which suggests that either Q-peptides should be predominantly drawn from arginine terminated peptides, or alternatively, that a step such as guanidination should be used to convert lysine residues to homoarginine residues, enhancing the propensity to give strong signals (Brancia, Electrophoresis 22 (2001), 552-559). The QCAT was digested by trypsin very effectively and there was no evidence for partial proteolytic products of the Q-peptides, which would of course compromise the quantification step. The inventors then expressed the protein in minimal medium containing $^{15}NH_4Cl$ as sole nitrogen source. When digested with trypsin, the resultant MALDI-ToF mass spectrum was of high quality, and all Q-peptides were detectable at the appropriate mass shift corresponding to the number of nitrogen atoms in the peptide (data not shown).

[0049] The unlabelled and ^{15}N-labelled QCAT proteins were then mixed in different ratios, and digested with trypsin before the resultant limit peptides were analysed by MALDI-ToF mass spectrometry. The heavy and light variants of the peptides were readily discerned (Figure 3a) and their intensities measured for a series of peptides (Figure 3b). In all instances, the data were of high quality, and the relationship between proportion of material and the heavy:light ratios was linear, with a slope of one (mean $\pm$ SD (n=6) = 1.008 $\pm$ 0.008), and very high correlation coefficients (r^2 greater than 0.99 in all instances). The combined data (Figure 3c) expresses the data for seven peptides; the close boundaries defined by the 95% confidence limits indicate the quality of the quantification.

Summary of results

[0050] The inventors have applied this particular QCAT in the analysis of protein expression in chick skeletal muscle, at 1 d and 27 d post-hatching (Figure 4). Twelve proteins present in this preparation were also represented in the QCAT. MALDI-ToF data of the tryptic peptides was readily acquired, and the changes in protein levels that occur over the first three to four weeks post-hatching were determined.

[0051] Because the proteins were absolutely quantified, the inventors were able to express the proteins as nmol per g wet weight of tissue. The variance of the triplicate analyses was small; the inventors attribute this variance to biological rather than analytical variation. The inventors have previously measured the levels of seven of these proteins by 2D gel electrophoresis and densitometry, and the correlation between the quantification using both methods was 0.82 (r^2, p>0.001). Recognizing that the two methods assess different representations of the proteome, such as charge-variant isoforms or total protein complement, and that the densitometirc method is inevitably imprecise, the correlation is good.

[0052] The inventors have demonstrated the feasibility of the QCAT approach for generation of a concatenated set of Q-peptides. The QCAT, designed using both theoretical and experimental considerations, was expressed at high levels, even when grown on minimal medium, and the product was successfully purified. Because the QCAT is a completely artificial construct, the inventors did not anticipate that it would fold into any recognizable three-dimensional structure, and as expected, the protein aggregated into inclusion bodies. This is an advantage as subsequent purification is simpler, only requiring resolubilization of the pellet in strong chaotropes prior to affinity purification. Further, the lack of higher order structure of the QCAT would ensure that the QCAT was digested at least as quickly as the target proteins to be quantified.

Use of the artificial protein and the QCAT peptides

[0053] There are two ways in which the concatamers will be used. First, in the **direct Q-peptide** method, the concatamers are used as an internal standard. The stable-isotope labelled concatamer can be directly added to a sample or cell preparation before the proteolysis step. Alternatively, for some cell systems, the concatamer quantification can be used to achieve absolute quantification of a reference strain grown under carefully defined conditions - the **indirect Q-peptide** approach. This reference strain can then be used, in stable isotope labelled form, as an absolute quantification standard for all future proteomics quantification studies using that organism. Once a reference strain is accurately quantified, any peptide can be used to report on a protein, rather than the restricted set used as Q-peptides and this is clearly a very attractive proposition. This extends the generality of different proteomic strategies, and creates a new niche for tagging methods such as ICAT (Gygi, J Proteome Res 1 (2002), 47-54) and ITRAQ (Ross, Molecular and Cellular Proteomics in press (2004)) in a comparative proteomics analysis of an unknown against a fully quantified strain.

[0054] However, the inventors recognize that there are many instances where this approach is not appropriate, and where a stable isotope labelled concatamer itself (the **direct Q-peptide** approach) will be the appropriate standard. This is particularly apposite in proteomics studies using biological material that cannot be readily pre-labelled, for example, in animal tissues or in biomarker studies.

Particular advantages

[0055] The strategy the inventors advocate is superior to chemical synthesis of each individual Q-peptide, usually in a stable isotope form. Whilst chemical synthesis has been used in one-off applications, the process of peptide synthesis is not sufficiently 'clean' as to obviate exhaustive purification of the product. Secondly, for multiplexed assays, each peptide would need to be individually quantified before use. Finally, chemically synthesized Q-peptides are a finite resource whereas repeated expression of the QCAT gene is facile. High quality absolute quantification may be an effective route to overcome the difficulties associated with current methods for comparative proteomics, whether based on gel analysis or mass spectrometry. A series of comparative studies of particular cellular systems, each by comparison to a QCAT quantified reference would not only be individually quantified, but should be sufficiently rigorous that as the data sets grow, any pairwise comparison would be robust, transferable between individual laboratories and stable over time.

[0056] It should be possible to factor in the propensity of the peptide to ionize and generate a good signal in the mass spectrometer. At present, ion intensities are not used exhaustively in the analysis of mass spectra, although there have been some recent attempts to predict intensity using knowledge-based approaches (Krause, Anal Chem 71 (1999), 4160-4165; Gay, Proteomics 2 (2002), 1374-1391; Baumgart, Rapid Commun Mass Spectrom 18 (2004), 863-868).

[0057] An additional factor that must be taken into account is the choice of precursor label. Whilst uniform labeling with [^{13}C] or [^{15}N] ensures that every peptide is comprehensively labeled, it might be preferable to select Q-peptides that each contain the same amino acid that is then used as the stable isotope labeled precursor. Since most QCAT proteins would be anticipated to be assemblies of tryptic peptides, a strategy of incorporation of [^{13}C$_6$]-lysine and [^{13}C$_6$]-arginine would also ensure that most Q-peptides would be singly labeled and the mass offset between heavy and light peptides would be a constant 6 Da. Further, an unlabelled QCAT could be labeled *in vitro* using reagents advocated for comparative proteomics, enhancing all of these technologies to absolute quantification.

[0058] Without being bound by any particular theory, the inventors believe that the number of Q-peptides that could be assembled into a single QCAT is limited by the ability to achieve high-level heterologous expression of large proteins. In the example given here, the inventors chose 20 peptides of average length 15 amino acids and average molecular

weight 1.5 kDa. If 100 proteins were represented in a single QCAT, the resultant recombinant protein would be 150 kDa, which should be readily expressed. The entire yeast proteome, of approx 6000 proteins, could then be defined within approx. 60 QCAT constructs.

[0059] This ability to quantify as many as 100 proteins in a single construct invites the challenge of optimal assembly of individual Q-peptides in QCATs. Different criteria might be envisaged. First, a group of Q-peptides would allow absolute quantification of a particular subcellular fraction, or of a specific subset of proteins, for example transcription factors or protein kinases. Secondly, and perhaps more importantly, concatenation could be driven by the abundance of the target proteins in the cell. It would be difficult to quantify two different proteins with widely different expression levels in the same QCAT experiment, and it might be preferable to assemble high abundance proteins in a construct distinct from that encoding low abundance proteins.

[0060] Other applications for QCATs are readily envisaged, particularly in the broad areas of clinical biology and toxicology and diagnostics. Absolute quantification will add a new dimension to the predictive values of analyses in clinical or other biomarker monitoring systems and will absolutely define the stoichiometric ratios of individual proteins within a subcellular compartment or a multi-protein complex.

Materials and methods

[0061] **Materials**. [^{15}N]H$_4$Cl (99% atom percent excess) was provided by CK Gas Products Ltd. Hampshire, UK. Most reagents, except where listed here, have been described previously (Doherty, Proteomics 4 (2004) 2082-2093; Pratt, Proteomics 2 (2002), 157-160). Chick (layer, Hi-Sex Brown) skeletal muscle proteins were prepared from 1 d and 27 d old chicks as a 20.000g supernatant of a 10 % (w/v) homogenate (Doherty, Proteomics 4 (2004) 2082-2093; Doherty, Proteomics 5 (2005) 5, 522-533.

[0062] **QCAT gene design and construction.** Q-peptides were selected for uniqueness of mass, propensity to ionise and be detectable in mass spectrometry, the presence of specific amino acid residues (for example, leucine or valine), the absence of other amino acid residues (cysteine, histidine, methionine). The peptide sequences were then randomly concatenated *in silico* and used to direct the design of a gene, codon-optimised for expression in *E. coli.* The predicted transcript was analysed for RNA secondary structure that might diminish expression, and if this was present, the order of the peptides was altered. N-and C-terminal sequences were added as sacrificial structures, protecting the assembly of true Q-peptides from exoproteolytic attack during expression. Additional peptide sequences were added to provide an initiator methionine and a C-terminal cysteine residue for quantification. The artificial gene was synthesised *de novo* (by Entelechon GmbH, Germany) from a series of overlapping oligonucleotides, verified by DNA sequencing and ligated into the *Nde*I and *Hind* III sites of the pET21 a expression vector, to yield the QCAT plasmid, pET21a QCAT. A His$_6$ purification tag was provided by fusion to the vector.

[0063] **QCAT gene expression and labeling with ^{15}N.** The QCAT plasmid, pET21a QCAT, was used to transform NovaBlue (DE3) (K-12 *endA1, hsdR17(r_{K12}-m_{K12}+$), supE44, thi-1, recA1, gyrA96, relA1, lac,* F'[*proA*+B+, *lacIqZ□M15*:: Tn*10*(Tc^R)] cells to ampicillin resistance. Cells were grown at 37 °C in Luria broth, 100 μg/ml ampicillin to an A$_{600}$ of 0.4-0.6 and IPTG added to 1 mM. Incubation continued for a further five hours when cells were pelleted by centrifugation (5.000g, 4 min., 4°C), resuspended in 10 ml 20 mMTris/HCl buffer, pH 8.0 and lysozyme was added (100 μg/ml) for ten minutes at room temperature. Cells were then sonicated (three bursts of 30s) on ice and centrifuged at 14000 g for 10min. Pellets and supernatants of induced and uninduced cultures were analysed by 12,5 % (w/v) SDS PAGE/Coomassie blue staining. For ^{15}N-labelling, cells were grown in M9 minimal medium prepared using [^{15}N]H$_4$Cl (20 mM), and induced and processed as above.

[0064] **Purification and analysis of the QCAT protein.** The pellets from sonicated cells were dissolved in 20 mM phosphate buffer (pH 7.5) containing 20 mM imidazole and 8 M urea (Buffer A) before being applied to a NiNTA column (GE Healthcare). After 10 column volumes of washing in the same buffer, the bound material was eluted with buffer A with an increased concentration of imidazole (500 mM). This material was desalted on Sephadex G25 'spun columns' and the mass of the eluted protein was determined by electrospray ionisation mass spectrometry using a Waters-Micromass Q-ToF micro mass spectrometer. The mass spectra were processed using the MaxEnt I algorithm. The purified desalted protein was digested with trypsin, and the resultant peptides were mass measured using a Waters-Micromass MALDI-ToF mass spectrometer (Doherty, Proteomics 4 (2004) 2082-2093). To assess the response ratio of heavy and light variants of the QCAT, the purified 'heavy' and 'light' proteins were mixed in different ratios prior to digestion with trypsin and MALDI-ToF mass spectrometry. The intensities of the [^{14}N]- and [^{15}N]- peptides were measured on centroided spectra.

[0065] **Use of the QCAT to quantify muscle protein expression.** The supernatant fraction containing chicken soluble proteins derived from 100mg of tissue was mixed with 290 μg of [^{15}N] QCAT, quantified by protein assay and digested with trypsin overnight - the QCAT was digested at a higher rate than endogenous muscle proteins (results not shown). The experiment was replicated for three animals at each time point. Subsequently, the [^{14}N]- (muscle) and [^{15}N]- (QCAT) peptides were identified by mass, and their relative intensities measured by MALDI-ToF mass spectrom-

etry.

Mass Spectrometric Analysis

[0066]   The analysis in this example was carried out using a MALDI-ToF Mass Spectrometer, but this method is equally applicable to all other Mass Spectrometric methods suitable for the analysis of peptides.

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[0067]

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WO 03/102220 (Aebersold, R.)

SEQUENCE LISTING

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Claims

1. Artificial protein generated by gene design *de novo* comprising concatamers of Q-peptides which are signature peptides, generated by any proteolytic or chemical fragmentation for quantitative analysis of the proteome of a sample, cell or organism, comprising:

   (a) at least two consecutive peptides linked by a cleavage sequence for separating the peptides;
   (b) a singular marker on one or more peptides for determination of the absolute amount of the protein; and
   (c) N-terminal and C-terminal extensions for protection, purification and quantification of the peptides;

   wherein each peptide represents one single protein of the sample, cell or organism, the peptide is unique within the set of Q-peptides and each peptide is in a defined stoichiometry, and wherein the protein comprises 20 - 60 peptides.

2. Artificial protein of claim 1, wherein the protein consists of 60 peptides.

3. Artificial protein of claim 1 or 2, wherein the cleavage sequence is cleaved by a protease, preferably by trypsin.

4. Artificial protein of any of the preceding claims, wherein the singular marker is a cysteine residue.

5. Artificial protein of any of the preceding claims, wherein one or more peptides are repeated identically one or more times, in order to achieve a particular stoichiometry between all peptide species.

6. Artificial protein of any of the preceding claims, wherein the protein comprises an affinity tag for purification of the protein.

7. Artificial protein of any of the preceding claims, wherein the protein is labelled by an isotope.

8. Artificial protein of any of the preceding claims, wherein each peptide comprises between about 3 and 40 amino acids, preferably about 15 amino acids.

9. Artificial protein of any of the preceding claims, wherein the peptides represent different conformational, metabolic or modification states of the protein.

10. Artificial protein of any of the preceding claims, wherein the peptides have a defined molecular weight distribution and quantitative ratios.

11. A collection of concatamers of Q-peptides defined in claim 1, which covers the complete proteome of an organism to allow the rapid quantification of the proteome of such an organism.

12. Vector comprising a nucleic acid encoding the artificial protein of any of claims 1-10.

13. Kit comprising the vector of claim 12 and/or the artificial protein of any of claims 1 -10.

14. A method for quantitative analysis of the proteome of a sample, cell or organism, comprising the steps of:

(a) quantifying the absolute amount of the artificial protein of any of claims 1-10 comprising concatamers of Q-peptides and N-terminal and C-terminal extensions for protection, purification and quantification or one peptide containing the singular marker;
(b) generating a preparation of the proteins to be quantified;
(c) mixing the products of steps (a) and (b);
(d) completely cleaving the artificial protein of any of claims 1-10 and the proteins to be quantified in step (b) at the cleavage sequence;
(e) determining the mass of peptides, and from this;
(f) calculating the absolute amount of each protein,

wherein the artificial protein and/or the peptides are isotopically labelled.

Patentansprüche

1. Künstliches Protein, das durch eine Gen Gestaltung *de novo* erzeugt wird, das Konkatamere aus Q-Peptiden umfasst, die Signaturpeptide sind, die durch irgendeine proteolytische oder chemische Fragmentierung erzeugt sind zur quantitativen Analyse des Proteoms einer Probe, einer Zelle oder eines Organismus, umfassend:

(a) mindestens zwei aufeinander folgende Peptide, die durch eine Schnittsequenz zum Trennen der Peptide verknüpft sind;
(b) eine einzelne Markierung auf einem oder mehreren Peptid/en zur Bestimmung der absoluten Menge des Proteins; und
(c) N-terminale und C-terminale Verlängerungen zum Schutz, zur Reinigung und zur Quantifizierung der Peptide;

wobei jedes Peptid ein einziges Protein der Probe, der Zelle oder des Organismus darstellt, das Peptid innerhalb der Reihe von Q-Peptiden einzigartig ist und jedes Peptid in einer definierten Stöchiometrie vorliegt, und wobei das Protein 20-60 Peptide umfasst.

2. Künstliches Protein nach Anspruch 1, wobei das Protein aus 60 Peptiden besteht.

3. Künstliches Protein nach Anspruch 1 oder 2, wobei die Schnittsequenz durch eine Protease geschnitten wird, vorzugsweise durch Trypsin.

4. Künstliches Protein nach einem der vorhergehenden Ansprüche, wobei die einzelne Markierung ein Cysteinrest ist.

5. Künstliches Protein nach einem der vorhergehenden Ansprüche, wobei ein oder mehrere Peptid/e ein oder mehrere Male identisch wiederholt sind, um eine besondere Stöchiometrie zwischen allen Peptidarten zu erreichen.

6. Künstliches Protein nach einem der vorhergehenden Ansprüche, wobei das Protein eine Affinitätsmarkierung zur Reinigung des Proteins umfasst.

7. Künstliches Protein nach einem der vorhergehenden Ansprüche, wobei das Protein durch ein Isotop markiert ist.

8. Künstliches Protein nach einem der vorhergehenden Ansprüche, wobei jedes Peptid etwa zwischen 3 und 40 Aminosäuren, vorzugsweise etwa 15 Aminosäuren umfasst.

9. Künstliches Protein nach einem der vorhergehenden Ansprüche, wobei die Peptide unterschiedliche konformative, metabolische oder modifikatorische Zustände des Proteins darstellen.

10. Künstliches Protein nach einem der vorhergehenden Ansprüche, wobei die Peptide eine definierte Verteilung des Molekulargewichts und quantitative Verhältnisse aufweisen.

11. Sammlung von Konkatameren aus Q-Peptiden, wie in Anspruch 1 definiert, die das vollständige Proteom eines Organismus abdeckt, um die schnelle Quantifizierung des Proteoms eines solchen Organismus zu ermöglichen.

12. Vektor, umfassend eine Nukleinsäure, die das künstliche Protein nach einem der Ansprüche 1 - 10 kodiert.

13. Kit, umfassend den Vektor nach Anspruch 12 und/oder das künstliche Protein nach einem der Ansprüche 1 - 10.

14. Verfahren zur quantitativen Analyse des Proteoms einer Probe, einer Zelle oder eines Organismus, umfassend die Schritte:

(a) Quantifizieren der absoluten Menge des künstlichen Proteins nach einem der Ansprüche 1 - 10, das Konkatamere aus Q-Peptiden und N-terminale und C-terminale Verlängerungen zum Schutz, zur Reinigung und zur Quantifizierung oder ein Peptid umfasst, das die einzelne Markierung enthält;
(b) Erzeugen einer Präparation des zu quantifizierenden Proteins;
(c) Mischen der Produkte der Schritte (a) und (b);
(d) vollständiges Schneiden des künstlichen Proteins nach einem der Ansprüche 1 - 10 und des in Schritt (b) zu quantifizierenden Proteins an der Schnittsequenz;
(e) Bestimmen der Masse der Peptide und daraus;
(f) Berechnen der absoluten Menge jedes Proteins,

wobei das künstliche Protein und/oder die Peptide isotopisch markiert sind.

Revendications

1. Protéine artificielle générée par conception de génes *de novo* comportant des concatamères de peptides Q qui sont des peptides avec signature, générés par n'importe quelle fragmentation protéolytique ou chimique pour l'analyse quantitative du protéome d'un échantillon, cellule ou organisme, comportant :

(a) au moins deux peptides consécutifs reliés par une séquence de clivage pour séparer les peptides ;
(b) un marqueur individuel sur un ou plusieurs peptides pour la détermination de la quantité absolue de la protéine ; et
(c) extensions N-terminales et C-terminales pour la protection, la purification et la quantification des peptides ;

dans laquelle chaque peptide représente une protéine individuelle de l'échantillon, cellule ou organisme, le peptide est unique dans la série de peptides Q et chaque peptide se trouve dans une stoechiométrie définie, et dans laquelle la protéine comporte 20 à 60 peptides.

2. Protéine artificielle selon la revendication 1, dans laquelle la protéine est constituée de 60 peptides.

3. Protéine artificielle selon la revendication 1 ou 2, dans laquelle la séquence de clivage est clivée par une protéase, de préférence par la trypsine.

4. Protéine artificielle selon l'une quelconque des revendications précédentes, dans laquelle le marqueur individuel est un résidu de cystéine.

5. Protéine artificielle selon l'une quelconque des revendications précédentes, dans laquelle un ou plusieurs peptides sont répétés une ou plusieurs fois d'une façon identique, afin d'obtenir une stoechiométrie particulière entre toutes les espèces de peptides.

6. Protéine artificielle selon l'une quelconque des revendications précédentes, dans laquelle la protéine comporte une étiquette d'affinité pour la purification de la protéine.

7. Protéine artificielle selon l'une quelconque des revendications précédentes, dans laquelle la protéine est marquée par un isotope.

8. Protéine artificielle selon l'une quelconque des revendications précédentes, dans laquelle chaque peptide comporte environ 3 à 40 amino-acides, de préférence environ 15 amino-acides.

9. Protéine artificielle selon l'une quelconque des revendications précédentes, dans laquelle les peptides représentent des différents états conformationnels, métaboliques ou de modification de la protéine.

10. Protéine artificielle selon l'une quelconque des revendications précédentes, dans laquelle les peptides ont une distribution du poids moléculaire et des rapports quantitatifs définis.

11. Un ensemble de concatamères de peptides Q tels qu'ils sont définis dans la revendication 1, qui recouvre le protéome complet d'un organisme pour permettre la quantification rapide du protéome d'un tel organisme.

12. Vecteur comportant un acide nucléique codant pour la protéine artificielle selon l'une quelconque des revendications 1 à 10.

13. Kit comportant le vecteur selon la revendication 12 et/ou la protéine artificielle selon l'une quelconque des revendications 1 à 10.

14. Une méthode pour l'analyse quantitative du protéome d'un échantillon, cellule ou organisme, comportant les phases de :

(a) quantification de la quantité absolue de la protéine artificielle selon l'une quelconque des revendications 1 à 10 comportant des concatamères des peptides Q ainsi que des extensions N-terminales et C-terminales pour la protection, la purification et la quantification d'un peptide contenant le marqueur individuel ;
(b) génération d'une préparation des protéines à quantifier ;
(c) mélange des produits des phases (a) et (b) ;
(d) clivage complet de la protéine artificielle selon l'une quelconque des revendications 1 à 10 et des protéines à quantifier dans la phase (b) à la séquence de segmentation ;
(e) détermination de la masse de peptides, et à partir de ceci ;
(f) calcul de la quantité absolue de chaque protéine ;

dans laquelle la protéine artificielle et/ou les peptides sont marqués isotopiquement.

Fig. 1

Selection of optimal peptides

Heterologous expression in *E. coli*

Absolute quantification of Q-cat

Unlabelled analyte

Mass spectrometric analysis of labelled Q-cat and unlabelled protein mixture, after proteolysis

Fig. 2

Fig. 3

a) GFLIDGYPR

100% L

82% L

64% L

12Da
54% L

44% L

23% L

0% L

b)

T3:GFLIDGYPR

T5:NLAPYSDELR

T8:QVVESAYEVIR

T9: LITGEQLGEIYR

T16: LVSWYDNEFGYSNR

T19:VLYPNDNFFEGK

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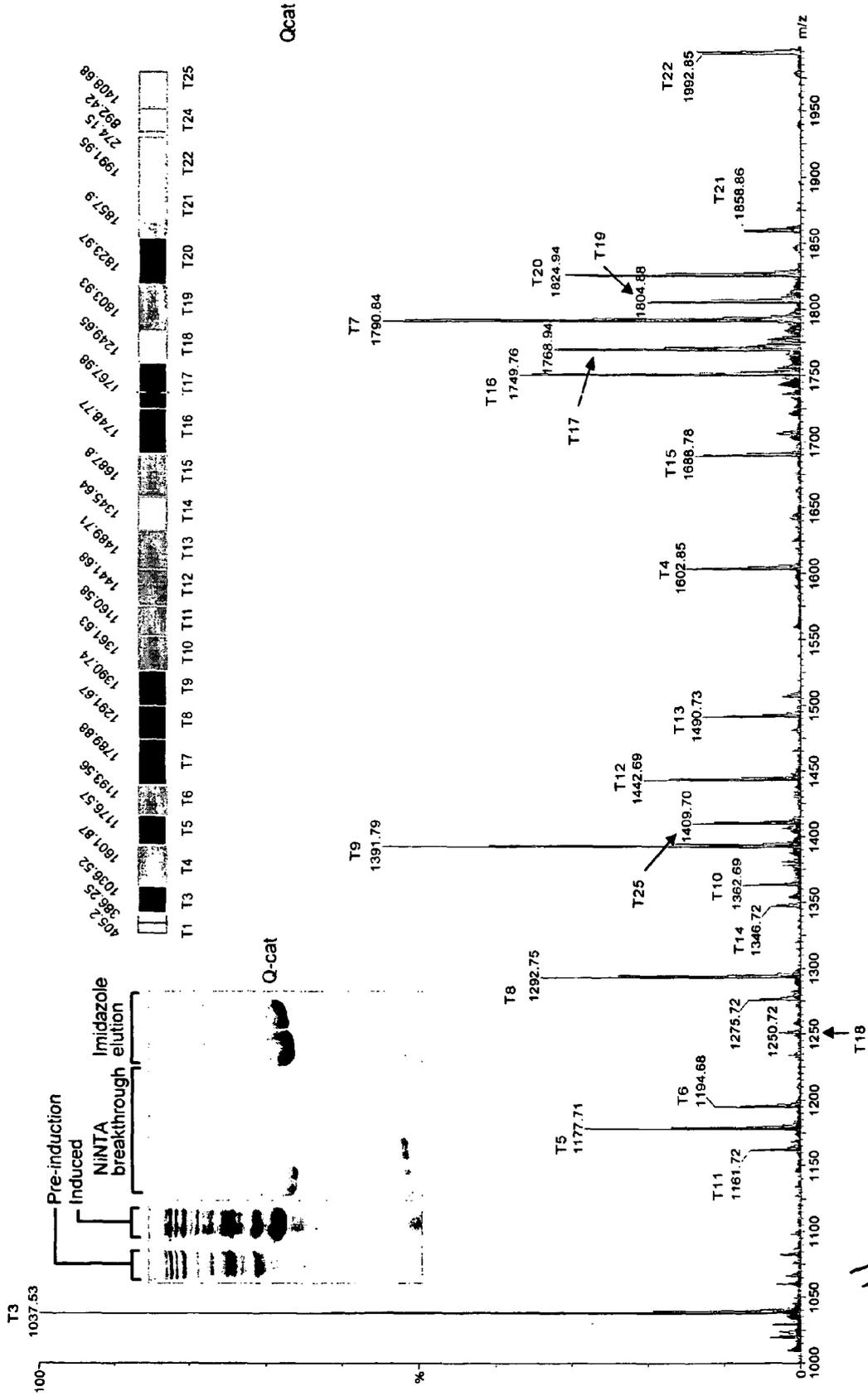
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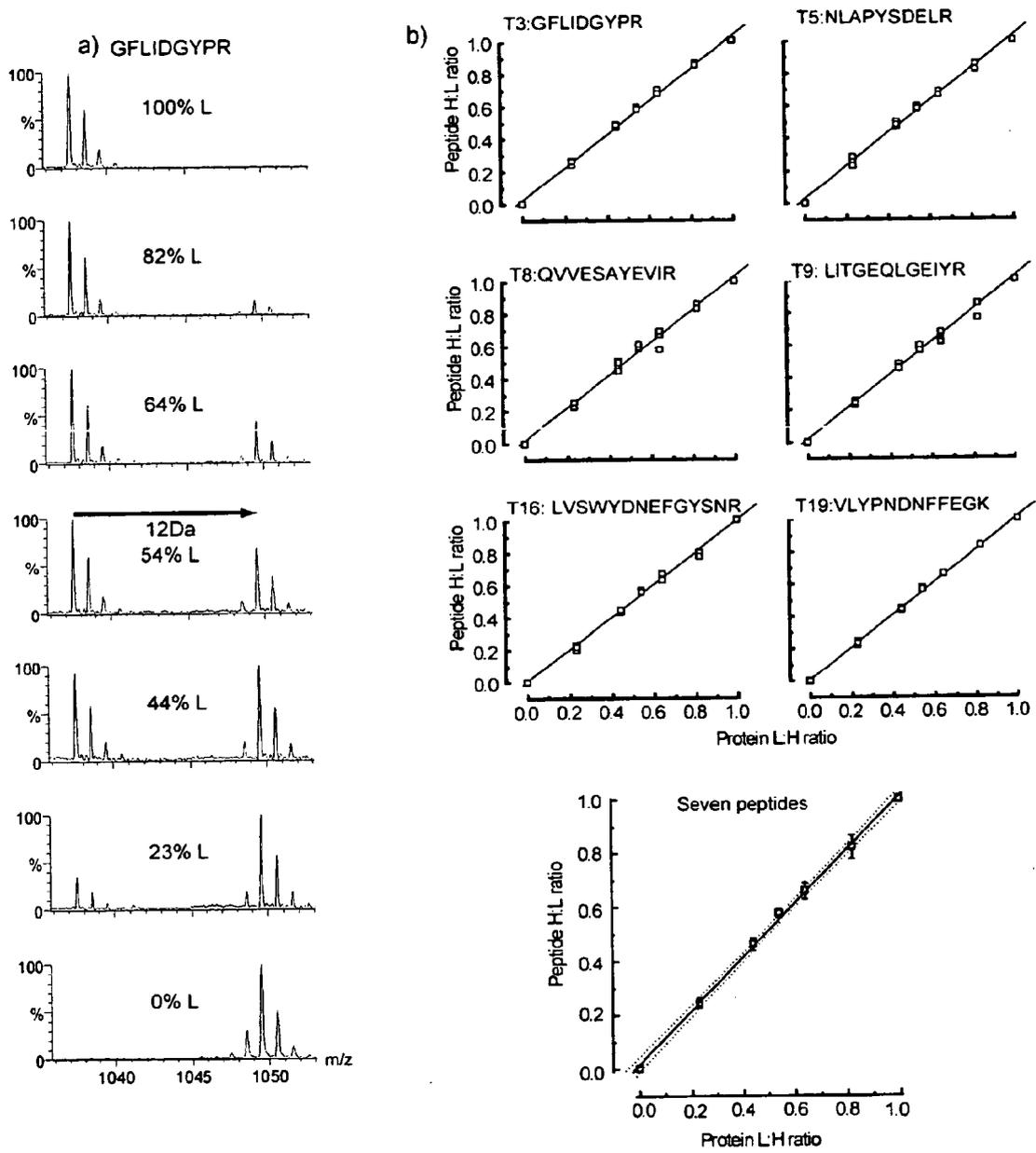
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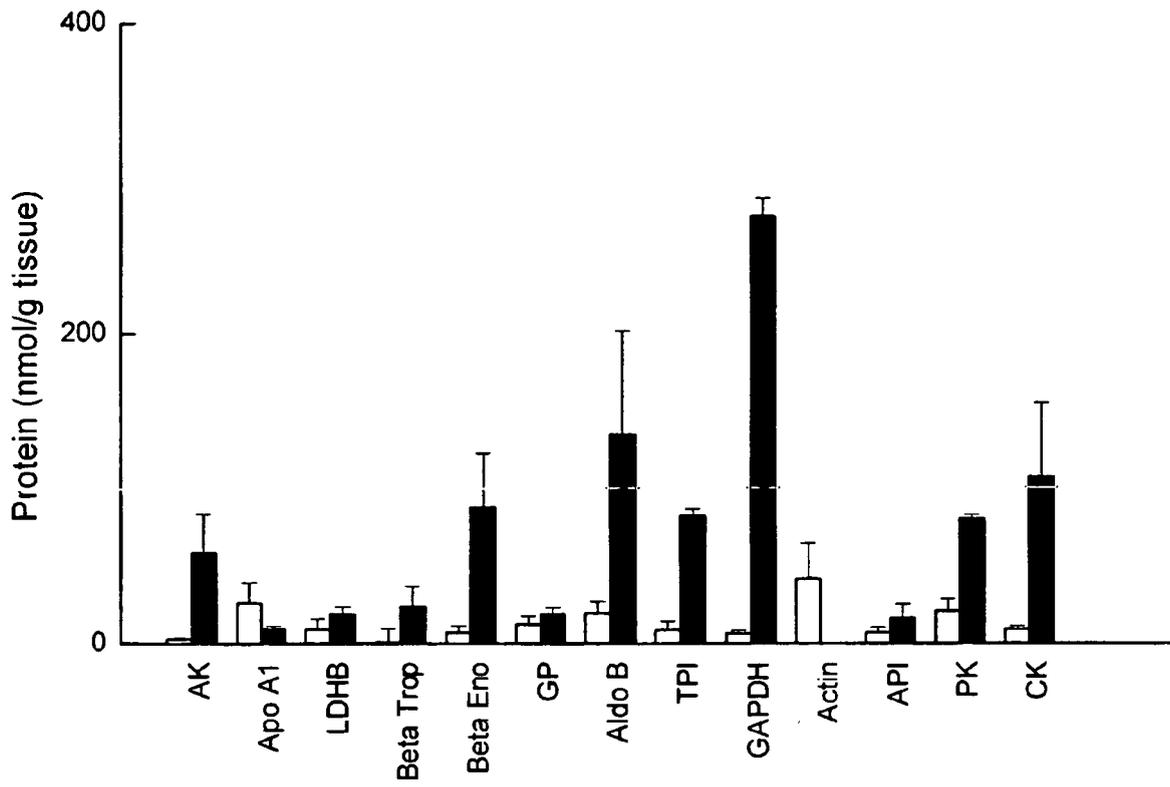
Protein L:H ratio

Fig. 4

Fig. 5

REFERENCES CITED IN THE DESCRIPTION

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