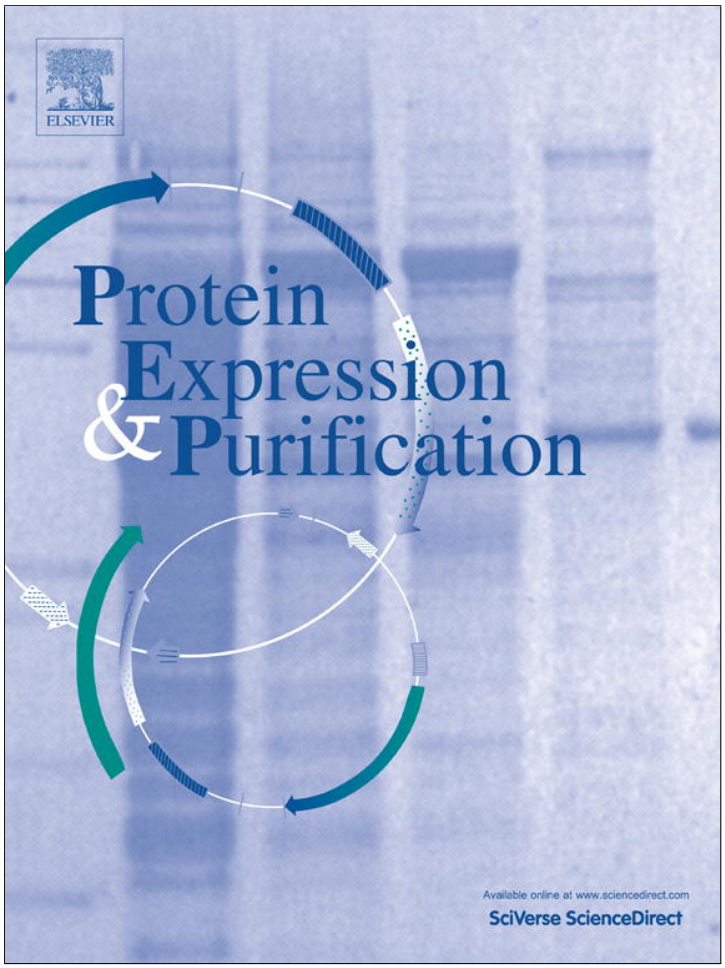

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Expression and characterization of ^{15}N-labeled human C-reactive protein in *Escherichia coli* and *Pichia pastoris* for use in isotope-dilution mass spectrometry

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ABSTRACT

Levels of C-reactive protein (CRP) in serum are correlated with inflammation and disease in humans. A higher level quantitative method, such as isotope-dilution mass spectrometry (ID–MS) is needed to compare and standardize the many commercial CRP assays. We compare the expression and purification of ^{15}N-CRP from *Escherichia coli* and *Pichia pastoris* and show that the protein isolated from *P. pastoris* has native pentameric structure along with high isotopic enrichment as shown by software developed specifically for this purpose. When this preparation was mixed in various ratios with unlabeled CRP and tryptic peptides of the mixtures were analyzed by LC–MS/MS, the ratios of heavy and light peaks were tightly correlated with input amounts of each protein. In this report we confirm the suitability of ^{15}N-rCRP as an internal standard in ID–MS. Standardization of CRP assays should help validate the relationship between CRP and human health.

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Introduction

C-reactive protein (CRP)[1] is a serum protein of pentameric structure released by the liver with a level in human serum that is diagnostically useful in the stratification of cardiovascular risk [1]. Multiple *in vitro* diagnostic assays are commercially available for clinical use although standardization of the results has been hampered by a lack of sufficient higher order analytic methods [2,3]. Isotope-dilution mass spectrometry (ID–MS) has been previously applied to the quantification of proteins using ^{15}N-labeled recombinant protein forms [4–9] as internal standards. The application of this method to the analysis of CRP requires the generation of ^{15}N-labeled CRP which, to this point, remains unavailable. Recombinant CRP (rCRP) has been successfully expressed using a number of expression organisms [10–12] and the current study sought to compare the generation of ^{15}N-labeled rCRP in *Escherichia coli* and *Pichia pastoris*. Bacterial heterologous protein expression has the benefit of producing large quantities of protein but often has severe limitations regarding protein solubility, folding structure and post-translational modifications-particularly disulfide bonds which are often crucial to correct protein folding. At the expense of lower yields, yeast expression systems offer the closest environment resembling the human cell for the nascent protein, including homologous chaperone proteins, non-reducing cytoplasm and secretion mechanisms. This study generated ^{15}N-labeled rCRP (^{15}N-rCRP) in both bacterial and eukaryotic expression systems for use as the internal standard in ID–MS with characterization including determination of the ^{15}N-incorporation percentage using custom software, the functionality in a structure dependent affinity binding and in the mass spectrometric response of tryptic-proteolyzed protein.

Materials and method

Plasmid construction for vector construction, transformation and expression in E. coli

A plasmid containing the human nucleotide sequence coding for CRP was obtained from GeneCopoeia (EX-GO194-B01) and amplified by polymerase chain reaction (PCR) using *Pfu* DNA polymerase (Promega) with appropriate primers (5′-GCA GAC AGA CAT GTC GAG GA-3′/5′-GTG AAG CTT TCA GGG CCA CAG CTG G-3′). The PCR product was subcloned into pET-45b(+) (Novagen) between the *PshAI* and *HindIII* sites (New England Biolabs) in-frame with the N-terminus hexahistidine coding sequence utilizing T4 DNA Quick ligase (New England Biolabs). Comparison of the translated construct sequence with the CRP protein sequence (entry P02741 in the ExPASy UniProtKB database) revealed a substitution ctg → ccg point mutation (L → P, amino acid 184) which was corrected by site-directed mutagenesis (QuikChange II XL – Stratagene) to produce the final expression vector, pCRPWT5 (primers: 5′-CAT GTG GGA CTT TGT GCT GTC ACC AGA TGA GAT TAA C-3′/5′-GTT AAT CTC ATC TGG TGA CAG CAC AAA GTC CCA CAT G-3′).

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[1] *Abbreviations used:* CRP, C-reactive protein; ID–MS, Isotope-dilution mass spectrometry; rCRP, Recombinant CRP; PCR, polymerase chain reaction.

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ArcticExpress (DE3) competent cells (Stratagene) were transformed with pCRPWT5 (50 ng) on M9 plates (M9 salts [13], 1 g/L NH_4Cl, 15 g/L agar, 4 g/L glucose, 2 mmol/L $MgSO_4$, 61.6 μmol/L $CaCl_2$, and 100 μg/mL ampicillin) and incubated at 37 °C overnight. Resulting colonies were inoculated into 150 mL of M9 medium as described above with the addition of 5 mg/L thiamine and 18.5 μmol/L $FeCl_3$ and incubated at 37 °C with shaking at 4.2 Hz (250 RPM) for 3.5 h and diluted into 3 L of fresh medium without ampicillin but substituting $^{15}NH_4Cl$ (Cambridge Isotope Laboratories). The cells were equally distributed into three 2.8-L baffled flasks and grown at 37 °C with shaking until the optical density at 600 nm (OD_{600}) reached 0.6 whereupon the incubation temperature was reduced to 16 °C. After stabilization, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a concentration of 100 μmol/L and expression was continued for 24 h with shaking at 4.2 Hz (250 RPM). Cell pellets were harvested, re-suspended in 100 mL of 100 mmol/L Tris–HCl (pH 8.5), 200 mmol/L NaCl, 2 mmol/L $CaCl_2$ and lysed by sonication (output 6, 50% duty, 2 min, 5 cycles). Lysed cells were centrifuged at 105,000 g_n for 60 min at 4 °C, and the resulting 100 mL supernatant was adjusted to 5 mM each of adenosine triphosphate (ATP) and $MgCl_2$ and applied to a 10 mL bed volume column of Ni^{2+} NTA resin (Qiagen). The column was washed (20 mL/h) with 10 bed volumes of the same buffer and then eluted with 60 mL of 300 mmol/L imidazole, 100 mmol/L Tris–HCl (pH 8.5), 200 mmol/L NaCl, 2 mmol/L $CaCl_2$ and collected in 30 fractions of 2 mL volume. The elution fractions were analyzed by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) and samples containing ^{15}N-rCRP were combined and further purified using *p*-aminophenylphosphorylcholine resin (Thermo Scientific). 30 mL of sample containing CRP was applied to a 10 mL bed volume column and washed (20 mL/h) with 6 bed volumes of buffer containing 100 mmol/L Tris–HCl (pH 8.5), 200 mmol/L NaCl, 2 mmol/L $CaCl_2$. Elution was performed with 60 mL of the same buffer but replacing $CaCl_2$ with 2 mmol/L ethylenediaminetetraacetic acid (EDTA) and collected in 30 fractions of 2 mL volume which were analyzed by SDS–PAGE. The appropriate CRP fractions were combined and the buffer was exchanged by dialysis to contain 10 mmol/L Tris–HCl (pH 8.5), 140 mmol/L NaCl, 2 mmol/L $CaCl_2$, 0.5 mg/mL NaN_3 and was stored at 4 °C. The purity of ^{15}N-rCRP was assessed by SDS–PAGE with Coomassie Blue and silver staining. Protein concentration was determined by Bradford assay (Bio-Rad) using bovine serum albumin as standard.

P. pastoris vector construction, transformation and expression

The portion of plasmid pCRPWT5 encompassing the nucleotide sequence encoding CRP was amplified by PCR using Phusion High-Fidelity DNA polymerase (New England Biolabs) (primers: 5′-TCT CTC GAG AAA AGA GAG GCT GAA GCT CAG ACA GAC ATG TCG AGG AAG G-3′/5′-AGG GAA TTC TCA GGG CCA CAG CTG GGG TTT GGT GAA CAC TTC GC-3′) in order to incorporate the *XhoI* and *EcoRI* restriction sites and delete the hexahistidine coding region. This insertion site will incorporate the α-Factor signal sequence of *Saccharomyces cerevisiae* into the expressed protein product. Vector construction, transformation and expression in *P. pastoris* were performed using the EasySelect Pichia Expression Kit (Invitrogen). PCR products were precipitated, digested (*XhoI* and *EcoRI*), purified and cloned into the like sites of plasmid pPIC9 (Invitrogen) to create plasmid pWLCRP. The pPIC9 vector includes the promoter for the alcohol oxidase gene (*AOX1*) which serves to induce the recombinant expression when methanol is used as the carbon source. The nucleotide sequence was confirmed by DNA sequencing. Creation of a CRP expressing *P. pastoris* strain was achieved by integrative transformation with plasmid pWLCRP, linearized with *SalI* to cut the *HIS4* coding region to target the *HIS4* locus of *P. pastoris* GS115 (His^-). The lithium chloride method was used for transformation, and the cells were placed on minimal dextrose (MD) agar plates (13.4 g/L yeast nitrogen base, 0.4 mg/L biotin, 20 g/L dextrose, 15 g/L agar) for the selection of His^+ transformants. The genotypes of His^+ transformants were verified by PCR. *P. pastoris* transformant cells were grown in 50 mL of buffered minimal glycerol medium (BMG – containing 100 mmol/L potassium phosphate – pH 6.0, 3.4 mL/L yeast nitrogen base without ammonium sulfate or amino acids, 10 g/L $(^{15}NH_4)_2SO_4$ (Cambridge Isotope Laboratories), 0.4 mg/L biotin, and 10 mL/L glycerol) overnight at 30 °C with shaking at 4.2 Hz (250 RPM). The overnight culture was diluted to 300 mL by addition of fresh BMG medium to an OD_{600} of 0.5 and grown to OD_{600} of 3 at 30 °C in 2.8-L baffled flasks. Cells were harvested by centrifugation at 3000 g_n for 10 min at room temperature, suspended in 1 L of induction medium (BMG with glycerol replaced by 5 mL/L methanol) in 2.8-L baffled flasks, and incubated at 30 °C with shaking at 3.2 Hz (190 RPM) for 5 days with daily additions of methanol (5 mL). The culture medium was separated from the cells by centrifugation at 7000 g_n for 15 min at 4 °C. Collected medium (approximately 1 L volume) was applied to 10 mL of *p*-aminophenylphosphorylcholine resin (Thermo Scientific) in one-step affinity purification with the same protocol, assay and storage conditions used in the bacterial expression.

Matrix-assisted laser desorption ionization mass spectrometry (MALDI MS) analysis

Intact protein analysis was performed on a Voyager STR-DE (AB Sciex) instrument in linear mode with delayed extraction and positive polarity. The accelerating voltage was 25,000 V with extraction delay time of 1700 nsec and a low mass gate of 4500 Da. 200 shots per spectrum were collected with a laser repetition rate of 20 Hz. Intact protein was diluted ten-fold in buffer (300 mL/L acetonitrile; 3 mL/L trifluoroacetic acid (TFA); balance water) and equally mixed with the same buffer containing 10 mg/mL sinapic acid. One microliter (1.3 pmol) of the prepared sample was spotted onto stainless steel target plates and allowed to air dry.

Tryptic peptide analysis was performed with a 4700 Proteomics Analyzer (AB Sciex) in reflector mode using default instrument mass calibration for MS and MS/MS analysis. CRP (43 pmol) was digested overnight at 37 °C with sequencing grade trypsin at a 1:50 ratio (mass fraction) in 25 mmol/L ammonium bicarbonate (pH 7.9). Digested sample was equally mixed with α–cyano-4-hydroxycinnamic acid (10 mg/ml) in buffer (400 mL/L acetonitrile; 1 mL/L trifluoroacetic acid (TFA); balance water) and was spotted (1 μL) onto stainless steel target plates and allowed to air dry.

Determination of ^{15}N-label incorporation level

The theoretical percentage of ^{15}N incorporation was determined by using software to match the MALDI–MS isotopic profile of tryptic peptides against probability profiles calculated using multinomial analysis [14]. A series of theoretical profiles having incremental ^{15}N incorporation rates was compared against the experimental profile in order to maximize the Pearson correlation coefficient (r) [15]. Criteria for selection of MALDI–MS spectra included signal-to-noise ratios greater than 200 and inclusion of five consecutive peaks from the isotopic envelope. The peak areas from four separate spectra of each peptide were averaged prior to incorporation analysis. The software is freely available for download from <http://www.nist.gov/mml/analytical/organic/isoenrichcalc.cfm>.

Table 1
Ion transition pairs of unlabeled and labeled CRP tryptic peptides used in LC–MS/MS analysis.

Peptide sequence	MS/MS transitions (precursor *m/z*/product *m/z*)	
	Unlabeled	Labeled
GYSIFSYATK	568.8/829.4	574.6/837.4
	568.8/916.5	574.6/925.5
YEVQGEVFTKPQLWP	911.0/853.4	920.4/862.4
	911.0/1016.6	920.4/1027.5
AFVFPK	354.7/391.2	358.2/395.2
	354.7/490.3	358.2/495.3
ESDTSYVSLK	564.8/609.4	570.3/615.3
	564.8/696.4	570.3/703.4
QDNEILIFWSK	696.9/793.5	704.3/801.4
	696.9/906.5	704.3 /915.5

High performance liquid chromatography tandem mass spectrometry (LC–MS/MS) analysis of digested CRP

Samples of purified human CRP (EMD Bioscience) were prepared containing 0.3, 1, 1.5, 2.0 or 2.7 μg of protein in 15 μL of buffer (25 mmol/L NH_4HCO_3, 1 mmol/L $CaCl_2$) containing 1 μg of BSA. To each tube was added 1 μg of ^{15}N-rCRP expressed by *P. pastoris* in 10 μL volume. The samples were denatured with heat and RapiGest surfactant (Waters), reduced with dithiothreitol (Sigma), alkylated with iodoacetamide (Sigma) and digested with trypsin overnight using the trypsin-to-protein ratio of 1:25 (mass fraction). The digested peptides were analyzed using an Applied Biosystems 4000 QTrap triple quadrupole mass spectrometer equipped with an Agilent 1200 series LC system. A C18 LC column (2.1 × 150 mm, 3 μm particle size, 30 nm pore size, Supelco) at 40 °C was used for the chromatographic separation of digested peptides. The "A" mobile phase consisted of 1 mL/L formic acid (FA) in water; the "B" mobile phase consisted of 1 mL/L FA in acetonitrile. A gradient elution was used, changing the A:B ratio of the mobile phases from 95:5 to 30:70 (volume fraction) in 15 min at a flow rate of 0.220 mL/min. Electrospray ionization in positive mode was used with multiple reaction monitoring to analyze ten transitions from five peptides shown in Table 1. The theoretical mass-to-charge ratio (m/z) of the labeled peptides and fragmented ions were determined using software available for download from <http://www.nist.gov/mml/analytical/organic/massfragcalc.cfm>.

Disclaimer

Certain commercial equipment or materials are identified in this paper in order to adequately describe the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standard and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Results and discussion

Bacterial expression and purification

The vector pCRPWT5 was sequenced to verify the encoding of the CRP protein (amino acids 19–224) without its native secretory signal sequence (amino acids 1–18) but including the aminoterminal hexahistidine tag (MAHHHHHHVGTGSNDDDDK). The vector was initially transformed into the *E. coli* bacterial strain BL21(DE3) and rCRP protein expression was induced. Multiple attempts at expression changing experimental conditions including time, temperature and IPTG concentration failed to produce soluble rCRP (data not shown). This vector was then transformed into the *E. coli* strain ArcticExpress (DE3) bacteria and induced with IPTG at 16 °C for 24 h using $^{15}NH_4Cl$ as the sole nitrogen source in the culture medium. Preliminary trials using inductions of 4 or 19 h indicated that the longer time was necessary for soluble protein expression. SDS–PAGE analysis shows in Fig. 1 – Panel A that the application of the soluble portion to a Ni^{2+}-NTA column shows significant enrichment of rCRP upon elution with imidazole. Further purification was obtained with a *p*-aminophenylphosphorylcholine column utilizing the binding affinity of the pentameric form of CRP for phosphorylcholine. Initial purifications of CRP in the current study included a significant contaminating protein band which was identified by in-gel digest analysis as bacterial chaperone protein (data not shown). The addition of ATP and Mg^{2+} was sufficient to remove the contamination in the current study consistent with previous reports that chaperone protein contamination may occur using the ArcticExpress bacteria and can be relieved by the supplementation of the purification buffer with ATP, Mg^{2+}, and K^+ [16]. SDS–PAGE analysis and Coomassie blue staining of the results shows in Fig. 1 – Panel B that highly purified rCRP (lanes E1 and E2) was obtained from the starting material (lane S). Further SDS–PAGE analysis of the combined fractions E1 and E2 indicates that greater than 98% of the silver staining is observed in the band associated with CRP. MALDI–MS analysis indicated that the mass of the protein is consistent with hexahistidine-tagged-^{15}N-labeled CRP with an assumed cleavage of the initiating methionine. A principle peak was observed of 25,367 mass-to-charge ratio (m/z) which closely matched the theoretical value (25,337 m/z) for this

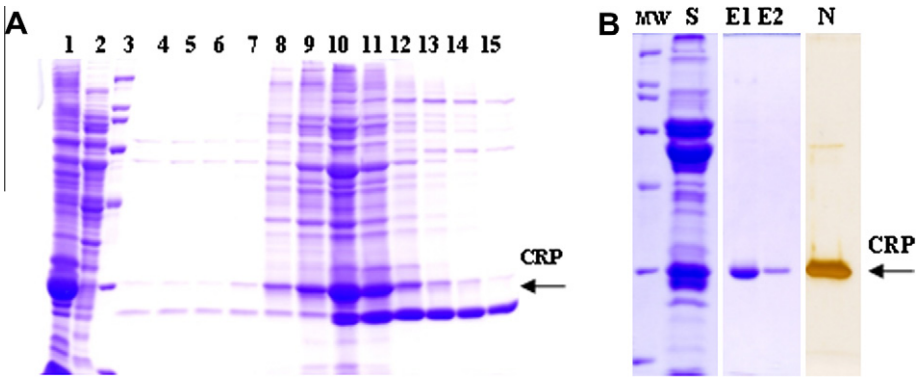

Fig. 1. Analysis of ^{15}N-labeled rCRP following expression in ArcticExpress (DE3) Panel A. Bacteria were lysed, fractionated and analyzed by SDS–PAGE with Coomassie Blue staining. Pellet and supernatant fractions are shown in lanes 1 and 2, respectively. The soluble lysate fraction was loaded onto a Ni^{2+}-NTA column, washed, and then eluted with imidazole. The initial elution fractions are shown in lanes 4–15. Panel B. The rCRP containing elution fractions were combined and applied to *p*-aminophenylphosphorylcholine resin and eluted with EDTA prior to a second SDS–PAGE analysis. The starting material is shown in lane S (20 μL loaded) and the two fractions containing eluted CRP are shown in lanes E1 and E2 (70 μL loaded). The elution fractions were combined for an additional SDS–PAGE followed by silver staining (lane N). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

structure. The production of ^{15}N-rCRP was extremely inefficient resulting in a production of only 20 μg of soluble CRP per liter of culture media. While this yield is too low to be practical, it nonethe-less indicates that production of soluble ^{15}N-rCRP was possible, leading to further efforts in eukaryotic expression systems.

Yeast expression and purification

This study sought to express rCRP in *P. pastoris* for the generation of ^{15}N-labeled protein. While recombinant CRP has been generated in cells from several eukaryotic species including *Spodoptera frugiperda, Trichoplusia ni, Kluyveromyces lactis and Leishmania tarentolae* [10,12], this is the first report of stable-isotope labeled protein expression. Eukaryotic systems benefit the heterologous expression of CRP by avoiding aggregation and protein mis-folding associated with bacterial systems, presumably due to the conservative nature of the chaperone proteins and similarities in cytoplasmic and organelle composition. Additionally, more efficient extracellular secretion has been demonstrated when using the *S. cerevisiae* α factor prepropeptide signal sequence [17]. Protein expression was induced by methanol addition in minimal medium containing $(^{15}NH_4)_2SO_4$ as the sole nitrogen source leading to the secretion of ^{15}N-labeled rCRP into the culture medium. The cells were removed from the medium which was directly applied to a *p*-aminophenylphosphorylcholine column and purified in a one-step process. Fig. 2 shows an SDS–PAGE analysis of the purification steps with Coomassie Blue staining. The elution fractions from Lanes 6 through 9 were combined to achieve a total yield of 1 mg of CRP (≈50-fold greater yield than with *E. coli*) with greater than 99% of the Coomassie staining associated with the CRP band. Mass determination of the eluted protein was performed by MALDI–MS which indicated the principle peak at 23,705 m/z instead of the 23,307 m/z expected for ^{15}N-rCRP. Such a large deviation could only be expected by the incorporation of additional amino acids into the protein. In fact, this mass difference is almost exactly equal to the mass of the sequence, EAEA, which is known to be incompletely processed from some proteins in this expression system [18–21]. Evidence for this is shown by the MALDI MS analysis of the aminoterminal tryptic peptide. As shown in Fig. 3, the peak at m/z 1151.49 exactly corresponds to that which would be found

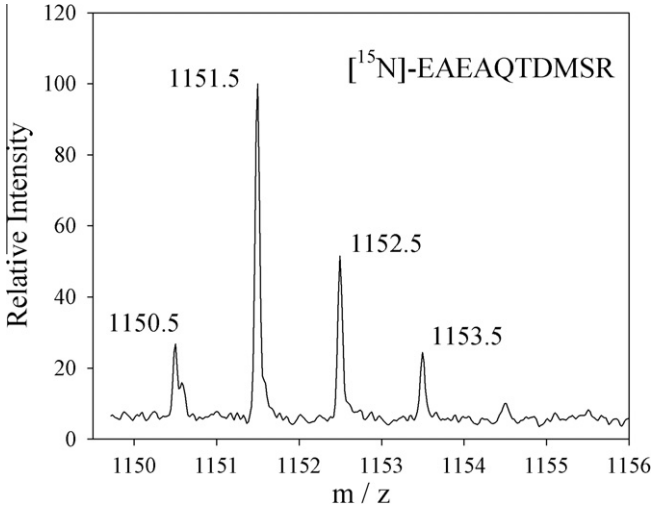

Fig. 3. MALDI–MS analysis of peptide EAEAQTDMSR. ^{15}N-rCRP was digested with trypsin and analyzed by MALDI–MS to determine the mass of the ^{15}N-labeled peptide EAEAQTDMSR shown with principle peak at 1151.5 m/z.

considering the additional sequences and isotopic ^{15}N-labeling. Further, MALDI MS/MS analysis of the sequence of this peptide also confirmed the addition of the EAEA sequence on the aminoterminus (data not shown).

Affinity purification of ^{15}N-rCRP

Binding to the pathogenic substrate, phosphorylcholine, and thus marking them for immunological response, appears to be the primary physiological role of CRP. This binding has been demonstrated only for the pentameric structure of CRP in the presence of calcium [22]. This study included calcium in all media and purification buffers to allow for cofactor incorporation. The structure of ^{15}N-rCRP was assessed by *p*-aminophenylphosphorylcholine binding. Both the *E. coli* and *P. pastoris* products were able to be specifically bound and eluted by withdrawal of calcium by EDTA. This

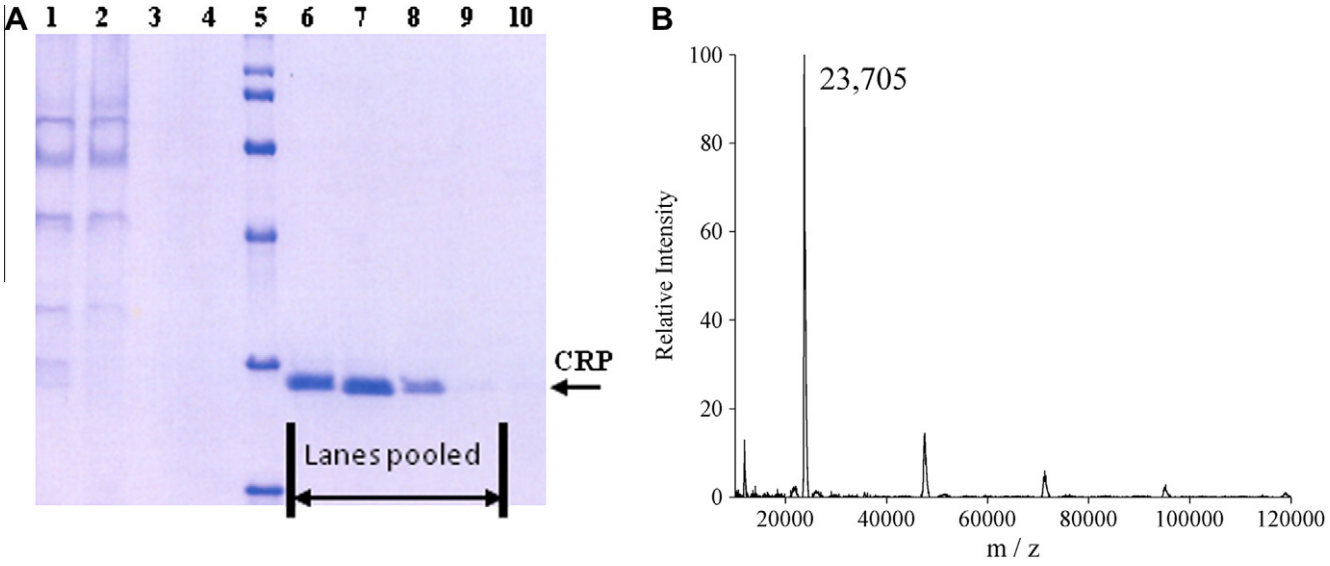

Fig. 2. Analysis of ^{15}N-labeled rCRP following expression in *P. pastoris* Culture medium (≈1 L) was removed from the cells by centrifugation and analyzed by SDS–PAGE. Panel A. Lane 1–100 μL of starting material; Lane 2 – flow through from *p*-aminophenylphosphorylcholine column application (100 μL); Lanes 3 and 4 – first and second EDTA elutions (100 μL); Lane 5 – protein molecular weight markers; Lane 6 – third EDTA elution (2 μL); Lane 7 – fourth EDTA elution (7 μL); Lane 8, 9 and 10 – fifth to seventh EDTA elutions (100 μL) Panel B. Elutions 6 – 9 were combined and were analyzed by MALDI–MS.

Table 2
^{15}N incorporation percentage of ^{15}N-rCRP tryptic peptides.

Sequence	m/z	Incorporation percentage	Pearson correlation coefficient	AA positions
EAEAQTDMSR	1151.4	98.3	0.9999	−4–6
GYSIFSYATK	1147.5	98.5	0.9985	48–57
QDNEILIFWSK	1407.7	98.1	0.9971	59–69
YEVQGEVFTKPQLWP	1839.9	97.7	0.9977	192–206

Numbering is based on sequence of mature secreted native CRP.

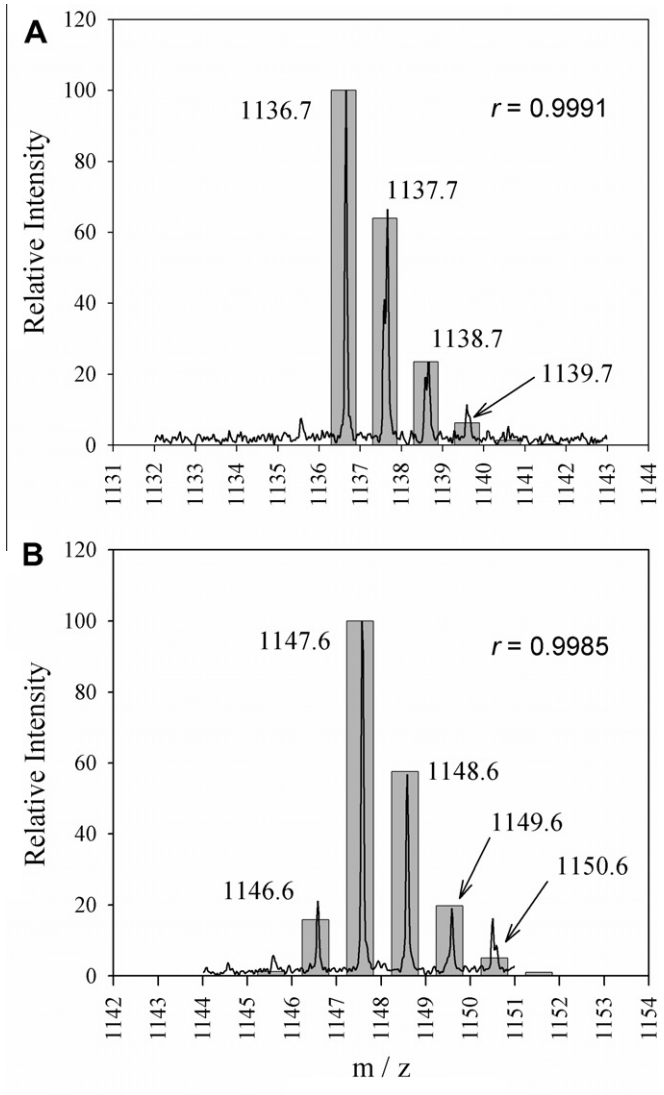

Fig. 4. Comparison of theoretical isotopic probability distribution versus peak areas from MALDI–MS spectra for the peptide GYSIFSYATK. Panel A – MALDI–MS of unlabeled peptide is shown (solid line) and overlaid with the theoretical distribution generated (gray column) for naturally occurring ^{15}N incorporation. Panel B – MALDI–MS of ^{15}N-labeled peptide is shown (solid line) and overlaid with the theoretical distribution generated (gray column) for the ^{15}N-labeled peptide based on 98.5% incorporation rate. The corresponding Pearson correlation coefficient is shown in each panel.

provides evidence of the correct association of the protein with the calcium cofactor as well as providing a straightforward means of purification of the rCRP from the cell culture media. The pentameric structure of the purified CRP can be assumed because other forms of rCRP will not be able to bind to the column. Additionally, misfolded protein which might occur due to incorrect disulfide bond formation would not be expected to bind correctly as well. As both the *E. coli* and *P. pastoris* forms appeared to bind and elute efficiently, the less favorable folding environment of the E. coli appears to have a greater effect on the expression efficiency as opposed to the structure of the purified form.

Percent incorporation of ^{15}N into P. pastoris rCRP

It is of particular interest to know the degree of ^{15}N incorporation of the protein, or tryptic peptide fragments, intended to be used as an internal standard so that there is not interference during MS measurement. The isotopic distribution profile of a peptide is quite sensitive to the abundance of its constituent heavy isotopes and as such is a good marker for ^{15}N incorporation. Using software created specifically for this task, the ^{15}N-incorporation was determined using four peptides from trypsin-digested rCRP from *P. pastoris* that were analyzed using MALDI–MS. The peptides are shown in Table 2 and include peptides encompassing the start and end of the expressed protein. The average ^{15}N incorporation percentage was 98.2% (CV = 0.3%) with an average Pearson correlation coefficient of 0.9986 (CV = 0.14%) indicating that use in ID–MS as an internal standard would be appropriate. Validation of the program was performed by correctly predicting the natural isotopic profile distribution of native CRP (r = 0.9991). Fig. 4 illustrates the match for the MALDI MS spectra for the peptide GYSIFSYATK against both the natural CRP profile (Panel A) and the ^{15}N rCRP labeled form (Panel B).

LC–MS/MS analysis of digested P. pastoris ^{15}N-rCRP

The ability of the ^{15}N-rCRP to produce linear responses within the mass spectrometer was assessed by digesting a series of samples of purified human CRP (ranging from 0.3 to 2 μg) each spiked with 1 μg of ^{15}N-rCRP. The ratios of the protein quantities, as well as their response ratios, is a true reflection of the intended use of

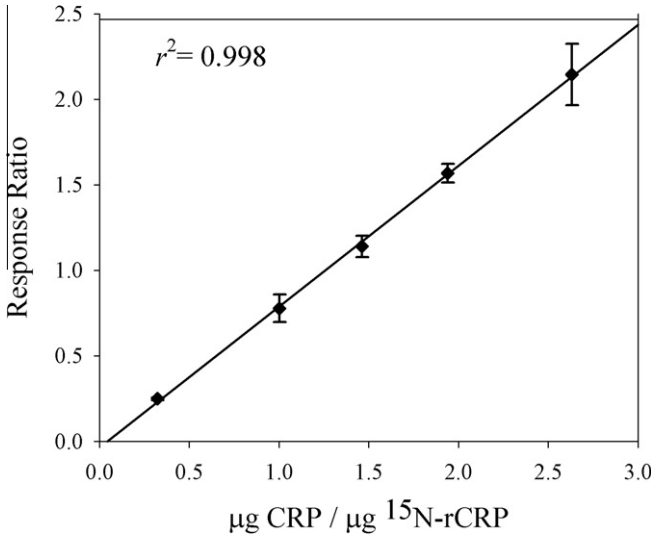

Fig. 5. Digestion and LC–MS/MS analysis of increasing quantities of purified human CRP with constant quantity of *P. pastoris* expressed ^{15}N-rCRP. Increasing amounts of purified human CRP (0.3, 1, 1.5, 2 and 2.7 μg) were combined with 1 μg of ^{15}N-rCRP (*P. pastoris* expression) and digested with trypsin prior to LC–MS/MS analysis. A total of 12 transitions from 5 peptides were monitored for both the labeled and natural forms. The response ratio was calculated by dividing the response area for the natural form by the corresponding response area of the labeled form. The response ratio of all the peptides were averaged at each amount and plotted against the corresponding mass ratio of natural-to-labeled CRP. Results are shown for three separate digestions from a common dilution set. Error bars indicate % coefficient of variation.

the labeled protein as an internal standard for which it must have a linear relationship to native CRP in the trypsin proteolysis and subsequent mass spectrometry. Each of the prepared samples was digested with trypsin and then analyzed by LC–MS/MS. The ratio of the mass of the unlabeled to labeled CRP was compared to the ratio of the peak response areas for each corresponding protein. As shown in Fig. 5, a linear relationship is demonstrated for the relationship of the ratios ($r^2 > 0.99$). The slope of the regression line would be expected to be equal to 1 in order to truly indicate that full digestion equivalency was achieved for the ^{15}N-rCRP. In this case however, the slope was slightly less than 1 with a value achieved of 0.8 and is likely due to the uncertainty associated with the stock concentrations of protein. However, this difference does not impact the use of ^{15}N-rCRP as an internal standard because of the strong linear relationship which exists to the native CRP. A common application of a labeled internal standard in ID–MS is to use as a constant volume spike. As long as the concentration of the spiked material is of appropriate scale to the expected value, the analysis will be normalized to the volume of the spike material and the actual concentration of the spike is not required for concentration assignment as long as the protein concentration of the standard CRP used for the calibration curve is accurate.

Use of the expressed ^{15}N-rCRP as an ID–MS internal standard provides the capability to normalize an analysis for a number of aspects such as recovery, digestion, volume transfers, MS ionization and instrument response drift. Accounting for these variables reduces measurement uncertainties in development of the quantification methods required for the generation of needed reference materials in the standardization of this important clinical analyte.

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