Removal of Histidine Residue From zn- α_2- Glycoprotein by Carboxypeptidase Enzyme Using Spectrofluorimetry and Maldi-TOF-Mass Spectroscopy

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Abstract: The present invention relates, in general, to methods developed of diagnosing and monitoring to binding with Zn-α_2-glycoprotein in order to designrational drugs. Zinc-alpha2-glycoprotein (ZAG) is a secreted protein that is present in most bodily fluids. It is interesting because of its ability to play many important functions in the human body, including fertilization and lipid mobilization. After the discovery of this molecule, during the last 5 decades, various studies have been documented on its structure and functions but still it is considered as a protein with an unknown function. Its expression is regulated by glucocorticoids. Due to its high sequence homology with lipid-mobilizing factor and high expression in cancer cachexia, it is considered as a novel adipokine. On the other hand, its X-ray crystal structure and folding structure is similar to MHC class I antigen-presenting molecule; hence ZAG may have a role in the expression of the immune response and providing defined binding-altered mutants for cellular signaling studies and potential medical application. Spectrofluorimetry and MALDI-TOF Mass Spectroscopy showed that ZAG binds DAUDA with *Kd*in the micromolar range. This study will examine removal of histidine residue from recombinant ZAG protein by enzymes through Spectrofluorimetry and MALDI-TOF Mass Spectroscopy.

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Introduction

Zn- α_2-glycoprotein (ZAG) is a 43 kDa soluble protein present in blood and many other bodily fluids such as serum, saliva, milk, sweat, urine, seminal and breast cyst fluids (Tada, Ohkubo et al. 1991). It was first identified and purified as an abundant protein in serum in 1961, present at a concentration of about 100mg/L (representing 0.2 % of total serum protein) (Burgi and Schmid 1961). Several studies have documented the structure and proposed biological role of ZAG but still strict function of this protein is unknown (Hassan, Waheed et al. 2008). A role for ZAG has been proposed in the regulation of body weight and age-dependent changes in genetically influenced obesity (Henshall, Horvath et al. 2006). There is no evidence for the direct role of ZAG in fertility but the sperm motility is a key factor to determine semen quality and its fertilizing capacity (Pal, Rajalakshmi et al. 2006; Ding, Qu et al. 2007). The ZAGis identical in amino acid sequence to tumor-derived lipid mobilizing factor (LMF), a protein associated with the dramatic loss of adipose body stores in cancer cachexia(Bing, Bao et al. 2004; Bao, Bing et al. 2005). The ZAG is overexpressesed during certain cancer cachexia and act as a cancer biomarker (Henshall, Horvath et al. 2006). Controlling ZAG/LMF's activity could be life-saving in the management of certain cancers and other cachexia inducing conditions (Bing, Bao et al. 2004; Henshall, Horvath et al. 2006). The crystal structure of human ZAG at 2.8 Å resolution revealed the resemblance of ZAG to heavy chain of major histocompatibility complex (MHC) class I molecules in overall folding and quaternary structure arrangement(Sanchez, Lopez-Otin et al. 1997; Sanchez, Chirino et al. 1999). A more refine crystal structure of the same protein was later reported by Delker et al. (Delker, West et al. 2004). The structure consisted of three domains (α1, α2, and α3) among which the α1-α2 superdomains adopt a similar folding pattern to immunoglobulin constant domains(Hassan, Waheed et al. 2008). These structures similarities of ZAG with MHC-I molecules reveal its possible role in immune responses (McDermott, Freel et al. 2006). The α3 is comprised of seven anti-parallel β-sheets containing extensive glycan chains proposing its functional implication in cell signaling(Hassan, Waheed et al. 2008). The presence of RGD sequence (Arg-Gly-Asp) at β4 strand of α3 domains reveals its cell surface receptors binding capabilities(Hassan, Waheed et al. 2008). The poly-histidine tag greatly facilitates the purification of recombinant proteins, however the presence of additional residues may induce subtle effects and compromises the biological activity of recombinant

protein (Araujo, Oliva et al. 2000). Researchers have employed different strategies for successful removal of histidine-tail resides (Arnau, Lauritzen et al. 2006). Here in this study, some experiments were performed to remove histidine tail reside from recombinant ZAG protein using carboxypeptidase Y and the evaluation of results was made by purifying with SDS PAGE and employing mass spectrometry (MS) and mass fluorometry techniques.

Materials and Methods

Protein expression

The commercially available recombinant vector (pET23) containing ZAG gene was transformed into E. coli cells for ZAG protein expression purposes. 200 mMIPTG was used for the induction of ZAG expression. The growth of overnight ZAG expression cells was measured at 600nm wavelength.

Protein purification

The pellet (1.48 g) of recombinant cells was suspended in 7.5 ml of Bugbuster protein extraction reagent to break the cell wall. 15µl of benzonase nuclease was added to reduce the viscosity of the lysate and contents were mixed on a Stuart roller mixer (SRT6D) at a slow setting for 20 minutes. The solution was centrifuged using Beckman J2 HS centrifuge at 16,000 x g, for 20minutes at 4°C. As the ZAG form inclusion bodies (McDermott, Freel et al. 2006) hence it should come in the pellet and therefore the supernatant was discarded. The pellet was washed in Bugbuster reagentusing the Novagen kit guidelines under the title: Inclusion Body Purification to remove the cellular debris. The purified pellet was reproduced by centrifugation at 10,000 x g at room temperature for 15 minutes in Eppendorf centrifuge 5804R. Finally, the pellet was dissolved in 25ml of 1 x binding buffercontaining 6M Guanidine hydrochloride (GdnHCl) to unfold the protein. The expressed protein was purified using His bind resin (Novagen) following the manufacturer's guidelines previously been followed by McDermott et al (McDermott, Freel et al. 2006). SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli(Laemmli 1970) protocol using 15 % polyacrylamide gel under reducing condition. ZAG was refolded in refolding buffer (0.1M Tris pH8, 2mM EDTA, pH8, 0.4M L-arginine hydrochloride, 0.5mM oxidised glutathione, 5mM reduced glutathione), kept over-night at 4 °C and then concentrated to a smaller volume using vivaspin 20 concentrators, where the content was passing through a detergent removal spin columns (Thermo Scientific) to remove further impurities. The UV absorbance of purified ZAG was recorded using an extinction coefficient ε□□□ = 68560$m^{-1}cm^{-1}$ in a Lambda35 UV/Vis spectrometer, Perkin Elmer in quartz cuvette of 1cm path length to estimate the amount.

Histidine tail removal

Carboxypeptidase (CY) enzyme was employed to attempt the removal of histidine residues from expressed ZAG protein. 1 mg/ml working standards range of Carboxypeptidase Y was prepared in 0.5 M sodium acetate buffer, pH 6.5 for these assays. Two experiments were carried out under different conditions. Experiment 1: Samples 1 to 5 were prepared and incubated at 37°C for carboxypeptidase Y digestion. Experiment 2: samples 6 to 10 were prepared and incubated at 25°C. The 15% SDS-PAGE page was run for evaluation of these samples.

Mass Spectrometry

The Bruker Autoflex MALDI TOF instrument was used for MS analysis during this work. Sinapic acid, 2,5-hydroxybenzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (CHCA) were used as the matrix. 1mg of albumin dissolved in trifluoroacetic acid (TFA) was used as the standard to calibrate the machine. 1µl of the different protein samples treated with carboxypeptidase Y enzyme was pipetted onto the target plate. Information on voltage- Ion source 1: 20.00kV, ion source 2: 17.80kV and lens: 7.42kV.

Fluorescence spectroscopy

Fluorescence measurement for determination of DAUDA binding to ZAG were recorded at 20 ^{0}C in a SPEX Fluo-Max flourometer (Spex Industries, Edison, NJ) using 2 mL samples in Phosphate-buffered saline (PBS; 171 mMNaCl, 3.3 mMKCl, 17 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2) in a silica cuvette. The excitation wave length used for DAUDA and intrinsic ZAG fluorescence are 345 and 290 nm, respectively(Araujo, Oliva et al. 2000; McDermott, Kennedy et al. 2002;Gorelsky, Basumallick et al. 2005).

Results and Discussion

Expression of Recombinant ZAG

The ZAG expression results were initially evaluated through SDS-PAGE (Figure 1). Though the ZAG was 32KDa glycoprotein but the bands highlighted by the red squares in Figure 1 indicate the presence of ZAG as it falls in between 25 and 35kDa ranges. However bands under the black squares in lanes 2 and 6 indicate the presence of high molecular weight impurities. A progress in purification was observed by removal of inclusion bodies as revealed in lane 6 of the SDS- PAGE (Figure 1). Furthermore purification was performed by refolding the ZAG in refolding buffer as revealed in SDS-PAGE image in Figure 2. The concentration of purified ZAG as determined by UV absorbance was 0.168mg/ml.

Affinity histidine tagremoval

Carboxypeptidase Y was employed to affinity histidine tag removal from recombinant ZAG protein. The carboxypeptidase Ysequentially cleave C-terminal amino acids from peptides and proteins (Jung, Ueno et al. 1999). The samples incubation at two different temperatures (25 and 37°C) and their 15% SDS-PAGE results revealed the carboxypeptidase Y digestion of C-terminus residues occurred best at 25°C (Figures 3, 4). Howeverexpected size difference for the removal of six histidine residues is very small and seems to be difficult to visualize on a SDS-PAGE.

MALDI TOF Mass Spectrometry (MS)

The molecular weight of one histidine is 155kDa, while the combine six histidine residues molecular weight is 930kDa. As a result of small size difference the simple SDS-PAGE cannot guided that how many histidine residues been removed during carboxypeptidase Y action. Therefore MALDI TOF *Mass Spectrometry* was carried out to get precise information regarding the number of histidine residues been cut off duringcarboxypeptidase Y action. The samples 6 to 10 incubated at 25°C for carboxypeptidase Y action showed an obvious size difference on SDS-PAGE. So these samples were run on the MALDI TOF MS for further confirmation. The overall MS results revealed size difference between uncut ZAG (sample 6) and cut ZAG (Samples 7-9) (Figure 5). Comparing the m/z values of cut and uncut ZAG corresponding to the highest intensity revealed the removal of approximately three histidine residues (Table 2).

Functional assessment of ZAG by DAUDA binding

The ZAG protein have the ability to bind fluorophore-tagged fatty acid 11-(dansylamino) undecanoic acid (DAUDA) (Kennedy, Heikema et al. 2001). This provides a fluorescence-based method for investigating ZAG-ligand binding and its functional aspects assessment. We used DAUDA and followed the florescence based method to perform the functional assessment of ZAG despite removal of its histidine residues. Our results indicated that despite CY digestion and removal of C-terminus histidine residues, ZAG can bind DAUDA and it is still functional (Figure 6).

ZAG protein. Lane 1**:** 5µl of Page ruler Prestained Protein Ladder; *lane 2:* 10µl solubilised GdnHCl-ZAG pellet; lane 3: 10µl of supernatant after incubation with GdnHCl-ZAG mixture; *lane 4:* 10µl of supernatant after incubation with 1x binding buffer; *lane 5:* 10µl of supernatant after incubation with 1x wash buffer; lane 6: 10µl of supernatant after incubation with 1x elute buffer. The binding buffer helps the ZAG bind to the resin whereas the wash buffer gets rid of any specifically bound impurities therefore no ZAG should be present in the supernatant as seen in lane 4 and 5. Elute buffer collects all the ZAG by separating it from the resin as revealed in lane 6, where low molecular weight inclusion bodies were eliminated during the process of purification.

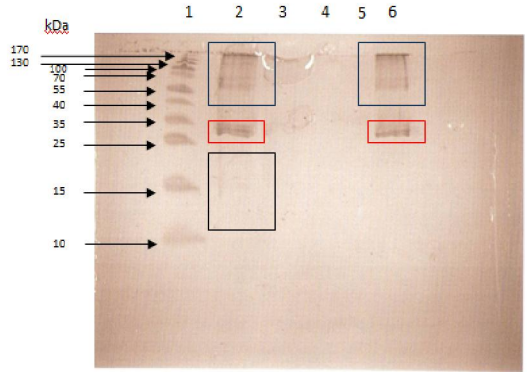


Figure 1: 15 % SDS-PAGE of cells lysate (containing expressed ZAG protein) & purified

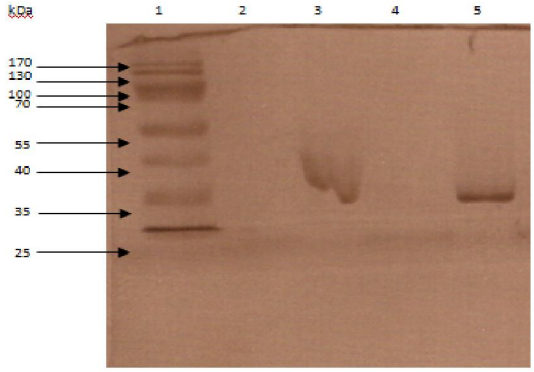


Figure 2:15% SDS-PAGE of purified refolded ZAG protein.

Lane 1: 5µl of Page ruler prestained Protein Ladder**;** lane 2: Empty**;** lane 3: 10µl of ZAG**;** lane 4: Empty**;** lane 5: 20µl of ZAG. The presence of ZAG can be confirmed from the bands in lane 3 and 5. In comparison to figure 1, it is more obvious that the impurities have been removed. Unfortunately due to the shape of the gel in lane 3 the band appears to be slightly bumpy in compare to lane 5.

Lane 1: 5µl of Prestained protein Marker Broad Range (Biolabs); lane 2: 10µl of sample 1 (50µl ZAG), lane 3: 10µl of sample 2 (50µl ZAG + 0.5µl CY); lane 4: 10µl of sample 3 (50µl ZAG + 5µl CY); lane 5: 10µl of sample 4 (50µl ZAG + 50µl CY); lane 6: 10µl of sample 5 (50µl CY). For these samples, no obvious size difference was observed between uncut ZAG (lane 2) and ZAG that was proposed to possibly cut with CY (i.e. lanes 3 to 5, highlighted with orange rectangle). CY has been shown to have run at its expected size of 64kDa indicated by the purple rectangle.

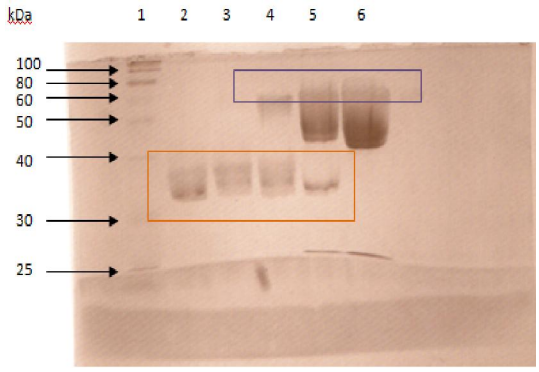


Figure 3: 15% SDS-PAGE of ZAG digestion with carboxypeptidase Y at 37°C.

5µl of Page Ruler Pre-stained Protein Ladder; lane 2: 10µl of sample 6 (50µl ZAG)**;** lane 3: 10µl of sample 7 (50µl ZAG + 0.5µl CY); lane 4: 10µl of sample 8 (50µl ZAG + 5µl CY)**;** lane 5: 10µl of sample 9 (50µl ZAG + 50µl CY)**;** lane 6: 10µl of sample 10 (50µl CY). In these samples.

Slight different electrophoresis pattern can observe for ZAG as compare to figure 3. Cut ZAG in lane 3 seems to be smaller in size than uncut ZAG in lane 2 indicating the probable removal of histidine residue/s from ZAG by the action of CY. In lane 3 and 4 no size difference can observe. It is because the difference in CY amount used in sample 7 and 8 is not large. However lane-5 (band indicated by an orange square) in comparison to the other slight size difference of ZAG can be observed. The obvious reason seems to be the larger amount of CY in sample 9 suggesting that the enzyme has performed further degradation of ZAG protein. The expected size difference to be seen for the removal of six histidine residues is very small to be visualized on a gel.

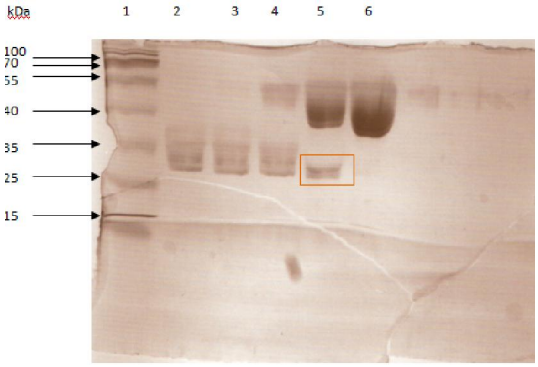
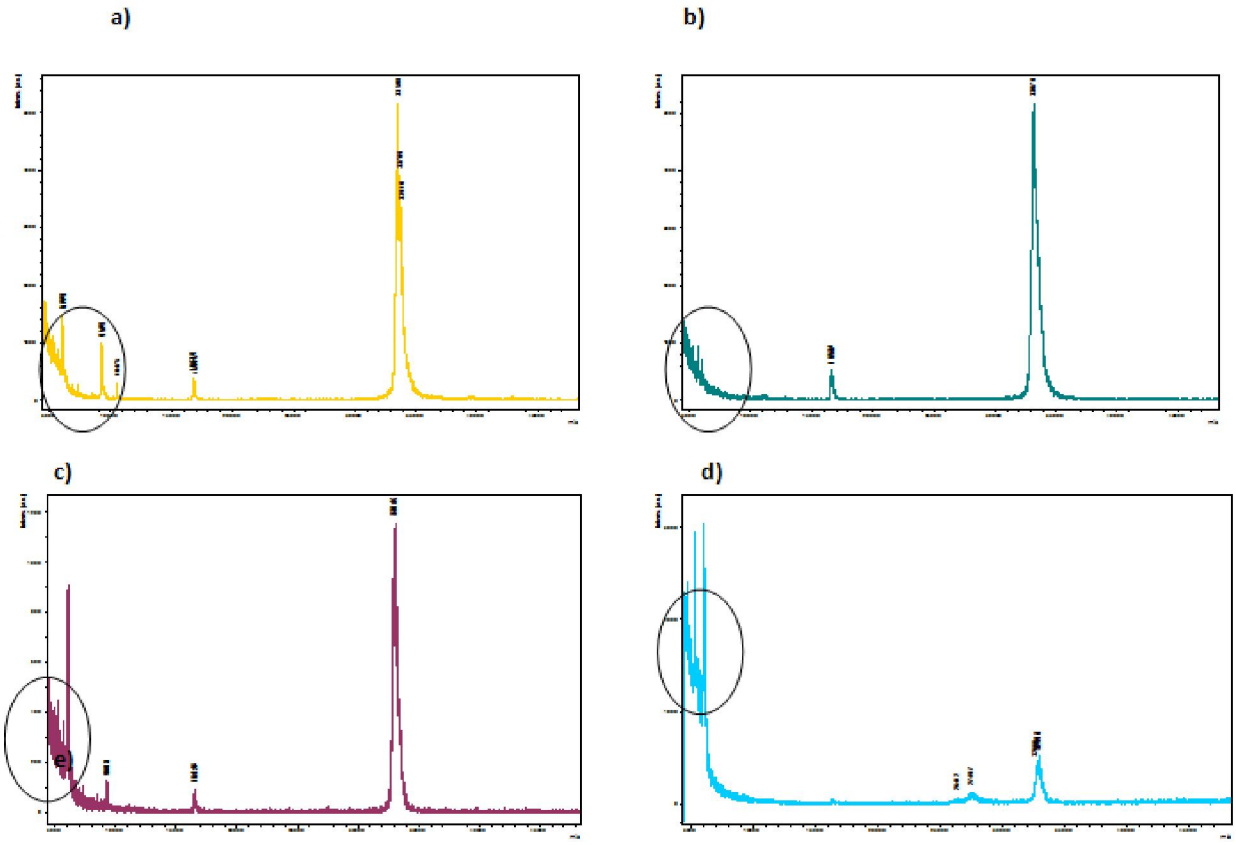


Figure 4: 15% SDS-PAGE of ZAG digestion with carboxypeptidase Y (CY) at 25°C. lane 1.



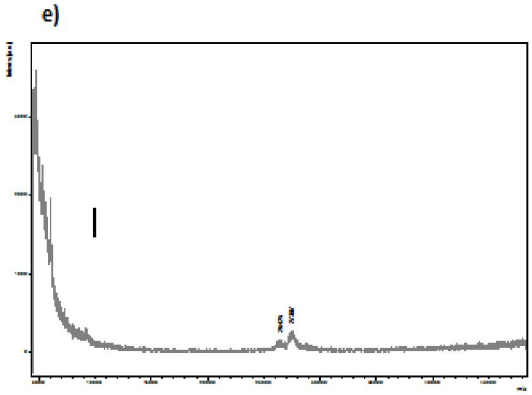


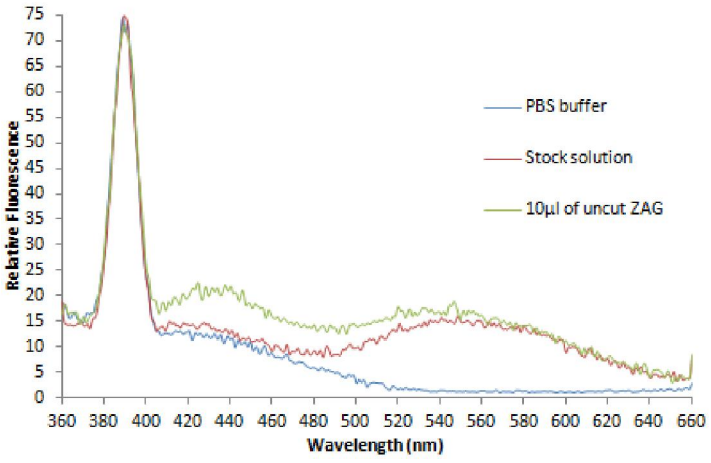
Figure 5: MALDI TOF MS results of samples incubated at 25°C for carboxypeptidase Y (CY).

The five graphs shown here representing MS spectra of ZAG alone, ZAG plus different CY concentrations and CY alone **(a)** Sample 6 consists of ZAG only representing by peak on the right with a m/z of 33508. Peaks on the left also represents ZAG but with two charges. The doubly charged ion forms at approximately half the m/z value. **(b)** Sample 7 consists of 50µl ZAG with 0.5µl CY represented by right and left peaks respectively. **(c)** Sample 8 consist of50µl ZAG with 5µl CY represented by right peak of 32947 m/z and left peak of 16446 m/z respectively. **(d)** Sample 9 consists of 50µl of ZAG with 50µl CY represented by the two small right and left peaks respectively. **(e)** Sample 10 contains 50µl of CY only seen by the presence of peak at 27387 m/z. Molecular weight of CY is 64kDa therefore peak at 27387 m/z represents CY with a 2+ charge.

Table 1: Them/z and intensity values of samples 6, 7 & 8. The difference in the two m/z values, i.e. 33508 and 33070 of samples 6 (uncut ZAG) and 7 (cut ZAG) respectively giving a value of 438. 930 that approximately representing the removal of three histidine residues.

	m/z	**Intensity**
Sample 6	16738	394.42
	16844	342.8
	16945	169.72
	33508	5017.83
	33709	3740.85
	33910	2316.93
Sample 7	16524	429.21
	16587	534.83
	33070	4282.36
Sample 8	16446	59.56
	16521	97.01
	32947	920.19
	33085	1108.89

(A)



(B)

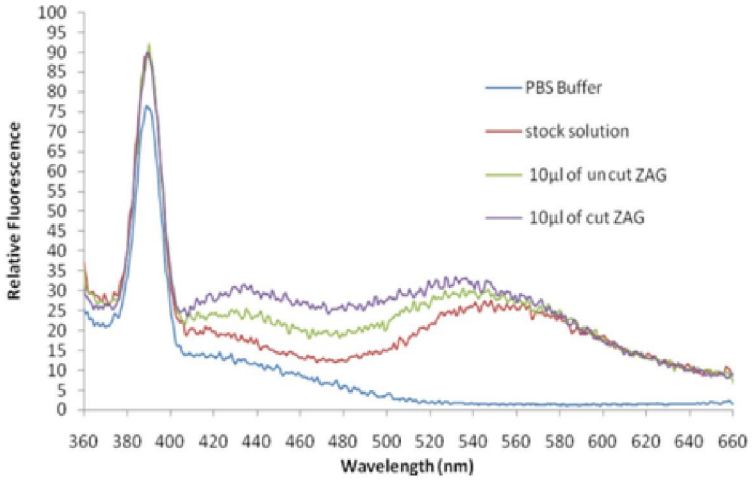


Figure 6: DAUDA binding and fluorescence analysis of ZAG before (A) and after digestion (B) with carboxypeptidase (CY) enzyme.

The stock solution contains 6µl of 1/10 DAUDA in 6ml PBS buffer. DAUDA fluoresces in buffer at around 545nm shown by red peak in both **A** and **B** (as identified by Kennedy et al 2001). In both **a** and**b** additions of 0.168mg/ml of ZAG caused the wavelength of DAUDA to decrease and a blue shift in fluorescence emission and intensity inductive of binding. In the uncut ZAG **(A)** wavelength of DAUDA changed from 545nm to 540nm upon binding of ZAG. In the cut ZAG **(B)** wavelength of DAUDA also changed from 545nm to 540nm and a further addition of ZAG changed DAUDA wavelength from approx 545nm to 535nm. Therefore change in wavelength and blue shift upon binding of ZAG showed that DAUDA bound to both cut and uncut ZAG samples and confirming that it is still functional after digestion with CY.

Conclusion

In conclusion our results showed that carboxypeptidase Y can employed for successful cleavage of c-terminus affinity histidine tag from recombinant Zn- α_2- glycoprotein without interrupting its functional aspects.

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