Characterisation of the active site of a newly-discovered and potentially significant post-proline cleaving endopeptidase called ZIP using LC-UV-MS

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Keywords

Liquid Chromatography, Mass Spectrometry, peptides, endopeptidase, enzyme.

Abbreviations

LC = liquid chromatography, MS = mass spectrometry, ZIP = Z-pro-prolinal-Insensitive-Peptidase, Z = N-benzyloxycarbonyl, UV = ultra violet, PO = prolyl oligopeptidase, PE = prolyl endopeptidase, LHRH = leuteinising hormone-releasing hormone, ESI = electrospray ionisation, ACN = acetonitrile, HPLC = high performance liquid chromatography, gly = glycine, pro = proline, leu = leucine, ala = alanine, met = methionine, phe = phenylalanine, ser = serine, tyr = tyrosine, his = histidine, glu = glutamic acid, MW = molecular weight, arg = arginine, K_i = inhibition constant, TIC = total ion current.

Abstract

There are enzymes that specifically recognise the amino acid proline within peptides and proteins that are called post-proline cleaving enzymes. Many of them are implicated in neurodegenerative disorders and psychiatric diseases. ZIP is a newly-discovered one of these peptidases. In this work, it has been purified from bovine serum and subjected to various analytical studies in order to characterise it. A series of reactions between synthesised peptides and ZIP were carried out in order to elucidate the size and specificity of the active site of the enzyme. On-line LC-MS was carried out on samples before and

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after incubation and the results obtained allowed us to detect if cleavage of the peptides was taking place, and if so, where in the peptide chain. It was found that the enzyme has a preference for another larger, bulky amino acid to follow the proline and that little or no cleavage was observed when polar acidic or other small amino acids were in that location. In terms of the size of the active site, the endopeptidase was found to have optimum activity when there were two more amino acids after the proline, with a fall-off in activity detected for the longer peptides. Data from kinetic studies confirmed the LC-MS results. The methodology described in this paper, which is a combination of LC separation and UV/MS detection, is required for the accurate monitoring of the reactions between the peptidase and its peptide substrates and for analysis of the products of such enzyme-peptide reactions. This work will assist in the design of site-directed inhibitors for new drug therapies.

INTRODUCTION

Enzymes are often very large in comparison to their substrate and the 3-dimensional active site is usually small, non-polar and specific for its substrate. In the active site, the enzyme converts the substrate to products by breaking (or making) chemical bonds. In the case of post-proline cleaving enzymes, the enzyme specificially recognizes the proline amino acid in the peptide or protein sequence and cleaves that peptide just after the proline, on its carboxyl side. One well-studied enzyme from this group is called prolyl oligopeptidase (PO)[1] or prolyl endopeptidase (PE), which is involved in the maturation and degradation of peptide hormones and neuropeptides[2]. It has been implicated in many disease states, especially depression[3], and the enzyme also has a role in the regulation of blood pressure[4]. In recent years however, a new and distinct post-proline cleaving endopeptidase, has been identified and reported[5,6]. This new enzyme has been designated ZIP (Z-pro-prolinal-Insensitive-Peptidase)[5] and preliminary investigation shows that the ZIP has the ability to cleave proline-containing peptides such as Substance P, LHRH, Angiotensin, Neurotensin and Bradykinin, and that it can be separated from PE by cation-exchange chromatography. Initial investigations on *human* serum have found it to contain very high levels of this ZIP activity so it is highly likely that it is actually this new ZIP enzyme (and *not* the reported PE) that shows significant activity changes in conditions such as depression, mania, schizophrenia and Alzheimers disease as reported by Maes and co-workers[3,7,8]**.** A recent paper has called for further exploration into the intriguing association between PE activity and affective disorders[9]. Clinical trials have been carried out in order to investigate the effects of both anti-

depressant and anti-psychotic drugs on serum PE levels but not, as yet, on the more significant ZIP levels. A number of important international pharmaceutical companies have been working on the design and chemical synthesis of highly specific PE inhibitors based on results from the inaccurate PE assay. Therefore, everything that can be learned about the binding and catalysis of the active site of this new ZIP enzyme will aid in the design of site-directed inhibitors.

The analysis of proteins and peptides is a rapidly growing field and many different analytical separation techniques are employed. Much characterisation work on enzymes and the necessary analyisis of peptides is carried out by using both chromatographic methods and spectrometric methods, often separate to each other e.g. collection of LC fractions from the column, sample preparation and subsequent injection of those samples onto a MS instrument[10]. However, tremendous progress in bioanalysis has been made by combining separation and identification into one technique in liquid chromatography-mass spectrometry (LC-MS). The most popular type of mass spectrometry used in LC-MS is electrospray ionisation (ESI) as it is readily compatible with LC. This technique has been gaining in popularity in recent years having been employed in the study and characterisation of antibodies[11], glycoproteins[12], histones[13], other proteins[14,15] and peptides[16,17,18,19,20]. In this paper, we describe a method to separate and characterise fragments of the reactions between this new ZIP enzyme and its substrates using LC-MS. We report on where cleavage takes place in the peptides and on the cleavage products formed. We demonstrate that LC-MS generates important analytical information about these enzyme-peptide reactions, and the technique has provided us with a convincing means to elucidate the structure and specificity of the active site of this new enzyme.

EXPERIMENTAL SECTION

Reagents and Chemicals

Spectranal grade acetonitrile (ACN) and and pestanal grade water, for use with the LC-MS, were supplied by Riedel-de-Haen. Formic acid (A.C.S. reagent) was purchased from Aldrich and was used for preparing mobile phase for the LC-MS system. The synthesised peptide substrates were obtained from the Royal College of Surgeons in Ireland. The standards Z-gly-pro-OH and Z-gly-leu-OH were obtained from Bachem. HPLC grade ammonium acetate and acetic acid used in the enzyme-peptide incubations were obtained from Fluka. HPLC grade ACN and methanol for use with the biological samples were obtained from Riedel-de-Haen.

Enzyme Preparation

Z-pro-prolinal-Insensitive-Peptidase (ZIP) was purified from bovine serum to near homogeneity by a combination of hydrophobic interaction, calcium phosphate cellulose, cibacron blue 3GA and finally S-300 gel filtration chromatography[6]. Active enzyme fractions were combined, concentrated ten-fold and then dialysed into 50mM ammonium acetate buffer, pH 7.2.

Sample Preparation

All of the peptide/standard substrates were prepared to 1mM concentration. Most of these were in 100% ammonium acetate buffer, pH 7.2. Where required, samples were initially dissolved in 20µl of methanol and then diluted to 1ml with the ammonium acetate buffer, pH 7.2, resulting in an overall sample matrix of 98/2 v/v ammonium acetate/methanol. For the incubates, 50µl of the enzyme (0.316 units/mg) in 50mM ammonium acetate buffer and 200µl of peptide (1mM) were mixed together. Hydrolysis of the peptides took place at 37°C over 24 hours. An enzyme blank sample was prepared by adding 50µl of ZIP in 50mM ammonium acetate buffer to 200µl of 50mM ammonium acetate buffer containing 2% methanol. The peptide blank samples were prepared by adding 50µl of 50mM ammonium acetate buffer only to 200µl of peptide (1mM). All blank samples were incubated under the same conditions. Reactions were quenched with 25µl of 50/45/5 v/v/v ACN/buffer/acetic acid.

For analysis by LC-MS, all samples were analysed neat except for the Z-gly-pro-OH standards which were diluted in order to produce a standard curve. The reason for replacing the usual phosphate buffer with ammonium acetate buffer, pH 7.2 and the usual trifluoroacetic acid quench with an ACN/acetic acid mix was to employ only volatile solvents for the electrospray ionisation. In order to examine the effect of the ZIP enzyme on analysis, samples were subjected to an ultrafiltration step in order to remove the enzyme prior to injection onto the LC-MS system. It was found that the enzyme did not interfere with any of the peptide or fragment peaks in either LC or MS traces, and in fact, the ultrafiltration membrane appeared only to retain some of the required sample components. Hence, ultrafiltration was not used as it was not required and would have compromised the recovery and hence the sensitivity of the method.

LC-MS Analysis

The instrument used was the Bruker/Hewlett-Packard Esquire LC - a Bruker mass spectrometer linked to a HP liquid chromatograph. The LC module of the instrument was a HP1100 with a variable wavelength detector, a low-volume pump, an in-line degasser and an autosampler. This LC system generally uses low flow rates, narrow bore columns and a micro flow cell in the UV detector. The MS module of the instrument comprised the ionisation chamber, the ion-trap to collect the ions and then to release them according to mass, and the ion detector to generate the spectrum. The Esquire-LC is capable of two types of ionisation - electrospray ionisation (ESI) and atmospheric pressure chemical ionisation, although in this work, we used only ESI. With ESI, samples are subjected to 'gentle' ionisation such that in-source fragmentation generally does not occur to any great extent. The LC isocratic method used a Zorbax RX-C18, 150 x 2.1mm, 5μm column with a mobile phase of 78/22/0.1 v/v/v water/ACN/formic acid at a flowrate of 0.15ml/min. The monitoring wavelength was 205nm and an injection volume of 4μl was used. After passing through the UV detector, the samples were introduced directly into the mass spectrometer, with the ESI source in the positive mode, enabling simultaneous UV and TIC (total ion current) traces to be obtained. The nebulisation gas and drying gas (both nitrogen) were set to 30psi and 8L/min respectively. The temperature of the source was maintained at 340ºC. Depending on the chosen target mass, the octopole voltage, skimmer 1 voltage and the trap drive voltage changed, but for mass 329 (sodiated Z-gly-pro) for example, these values were 2.48V, 34.4V and 32.5V respectively. Mass spectral data was generally collected in the scan range 50-1000 m/z.

Direct Infusion MS Analysis

Initially, incubate samples were directly infused into the MS source but some components of the mixtures were preferentially ionised over others which led to ambiguous results. Thus, it was decided to use LC-MS but the direct infusion analysis did aid in the development of the MS experimental conditions.

RESULTS AND DISCUSSION

LC Method Development

Column Selection

A number of reversed-phase LC columns were evaluated for this work (e.g phenyl, cyano and C8), but C18 was found to be superior in terms of resolving power. Reversed phase chromatography is the most widely used HPLC technique for the separation of peptides[21]. The type of C18 column required for this work was a Zorbax RX-C18, 150 x 2.1mm, 5μm. A longer column was also evaluated – Supelcosil C18, 250 x 2.1mm, 5μm – but the shorter 150mm Zorbax column gave improved chromatography in a faster time. The retention of peptides on reversed-phase columns often correlates well with their increasing relative hydrophobicities and C18-bonded columns, as opposed to the shorter alkyl chains (C4, C8), are recommended for the separation of smaller, hydrophilic peptides[21].

Choice of UV Wavelength

Peptide bonds absorb UV light strongly in the region 200-220nm. The wavelength of 205 nm was selected for the LC-UV work, as this was a reasonable compromise between sensitivity and selectivity, although in earlier work, where certain amino acids were being investigated, 200nm was also used. Hence LC-UV chromatograms obtained at these low wavelengths could be used to estimate relative yields of each cleavage product.

Mobile Phase Selection

Acetonitrile (ACN) was chosen as the organic solvent as it is highly volatile and relatively UV transparent. Various proportions of ACN were tried and tested until the isoctratic eluent of 78/22/0.1 v/v/v water/ACN/formic acid was found to be a good compromise between separation and speed. The incubate sample matrix was chosen such that the samples could be injected directly onto the LC-MS i.e. all solvents and acids were compatible (volatile) and did not interfere with the analysis.

MS Method Development

Direct infusion of samples at low flow rates facilitated on-line optimisation of the MS conditions. Various parameters were changed until the cleanest spectra with the highest intensity peaks were obtained. Inputting the target mass into the Bruker software (or the median target mass in the case of a range of analytes) automatically set certain parameters, although some of these settings e.g. octopole voltage, were changed and checked manually in order to further optimise the MS method. At all times, due consideration was taken of the fact that the LC methods developed would have to be compatible with the mass spectrometer.

Analytical Results & Discussion

Specificity of the Active Site

A number of peptides were obtained in order to examine the amino acid preferred after the proline by the ZIP enzyme (see Table 1). A range of amino acids with different polarities and sizes were selected. Figure 1 shows the UV chromatograms obtained before and after incubation for the peptides Z-gly-pro-tyr and Z-gly-pro-ser. Both indicate that some cleavage has occurred with the appearance of the Z-gly-pro fragment, but to a greater extent in the case of the Z-gly-pro-tyr peptide. Figure 2 shows the same post-incubation UV chromatogram for Z-gly-pro-tyr peptide superimposed onto the TIC (total ion current) trace, which allows us to 'see' the components of the incubation sample in two different ways using two different detectors – a UV detector and an MS detector. Figure 2 also displays the mass spectral data for the component peaks, confirming the identity of the compounds in each case.

The LC-MS results for the incubated samples showed that the greatest amount of cleavage in terms of formation of Z-gly-pro fragment, was obtained for the peptides containing amino acids with a higher molecular weight and a bulky R group after the proline. Little or no cleavage was observed when a polar acidic or a small amino acid was in that same position in the peptide (see Figure 3). These results agree to some extent with substrate specificity data for the PO enzyme where a preference for a hydrophobic residue was found when similar studies were carried out[1]. The kinetic experiments also concluded that the enzyme favoured a bulky residue after the proline (lowest K_i) and had a lower specificity for acidic and smaller residues such as glutamic acid, serine and alanine. These results correlated well with the results obtained by LC-MS.

The three amino acid residues that were found to give the most cleavage and the best kinetic data were phenylalanine, methionine and tyrosine. Two series of peptides containing phenylalanine and methionine after the proline respectively, were used as templates in the next experiment.

Size of the Active Site

Once the amino acid residue preference after proline was discovered, chain length effect could be investigated by increasing the number of residues after the proline. This was done in order to establish what effect if any, varying the size of the peptide would have on ZIP activity because it has long been known that the specificity of a peptidase is determined not only by the amino acids on each side of the cleavage point, but also on residues more distant from the point of hydrolysis. Two groups of peptides were prepared using Z-gly-pro-phe and Z-gly-pro-met as templates (see Table 2). The LC-MS results for the incubated samples showed that the greatest amount of cleavage i.e. formation of Z-gly-pro fragment, was obtained for the peptides with two further amino acids after the proline. As seen from Figure 4, production of Z-gly-pro decreased after this with increasing chain length. This indicates that the rate of hydrolysis of peptide substrates by ZIP decreases with chain length. This finding is supported by kinetic analysis where the inhibitor constants increase with chain length (data not shown). Figure 4 shows the UV chromatograms of the series of peptides based on Z-gly-pro-phe after incubation with ZIP enzyme and it is clear that the most cleavage occurs in the case of Z-gly-pro-phe-his peptide. Figure 5 shows the post-incubation UV chromatogram for Z-gly-pro-phe-his peptide and the mass spectral data for the three component peaks i.e. the parent peptide and both fragments, confirming the identity of the compounds in each case.

A similar trend was seen for the methionine series of peptides but with less cleavage overall than that evident in the phenylalanine series of peptides.

Proline Specific Peptidase Confirmation

It was decided to incubate two further synthetic peptides with the ZIP enzyme in order to confirm that the peptidase enzyme would not cleave peptides without proline in the sequence. Proline is an imino acid with a very unique cyclic structure and it was expected that any replacement of this residue would result in no cleavage by ZIP at all. To this end, a leucine amino acid was introduced into the proline position in the peptide chain yielding Z-gly-*leu*-phe-his-OH. This peptide was incubated for 24 hours

with ZIP in the same way as all other peptides. The presence of leu instead of pro had a drastic effect on the activity of ZIP towards the peptide and no cleavage was observed by LC-MS i.e. no fragments were produced and the parent peptide remained intact.

CONCLUSIONS

The peptidase enzyme ZIP has been incubated with two series of proline-containing peptides in order to characterise the specificity and size of the active site. We investigated in this work what effect the amino acid after the proline and what effect the chain length after the proline have on cleavage of proline-containing peptides by the enzyme. LC-MS has enabled us to separate and unambiguously identify the components of the biological reactions taking place and to confirm if the fragments obtained were the same as those predicted. The technique has proven to be very powerful for expedient and definitive determination of molecular weight of the products obtained in these reactions, allowing a better understanding of the size and specificity of the enzyme's active site. The data obtained helps us to understand the biochemistry of the enzyme-peptide reactions and to know more about the mode of action of the ZIP enzyme. The results indicate that the enzyme favours a bulky residue after the proline and in terms of chain length prefers two further amino acids after the proline, with ability to cleave decreasing with further elongation of the peptide. These results correlate strongly with kinetic data obtained for the same series of peptides.

Acknowledgements

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Table Captions and Tables

Table 1 : peptides chosen for investigation of preferred amino acid after the proline within the peptide.

Peptide sequence	Amino acid after proline	Polarity and/or charge of amino acid after proline	Size of amino acid after proline
Z-gly-pro-**ala**-OH	Alanine	**Non-polar, hydrophobic**	**MW 89**
Z-gly-pro-**met**-OH	Methionine	**Non-polar, hydrophobic**	**MW 149, sulphur in R group**
Z-gly-pro-**phe**-OH	Phenylalanine	**Non-polar, hydrophobic**	**MW 165, benzene ring in R group**
Z-gly-pro-**ser**-OH	Serine	**Polar, uncharged**	**MW 105**
Z-gly-pro-**tyr**-OH	Tyrosine	**Polar, uncharged**	**MW 181, benzene ring in R group**
Z-gly-pro-**his**-OH	Histidine	**Polar, basic**	**MW 155, five-membered ring in R group**
Z-gly-pro-**glu**-OH	Glutamic acid	**Polar, acidic**	**MW 147**

Table 2 : peptides chosen for examination of chain length effect on ZIP activity

Peptides based on Z-gly-pro-phe	Peptides based on Z-gly-pro-met
Z-gly-pro-**phe**-OH	Z-gly-pro-**met**-OH
Z-gly-pro-**phe**-his-OH	Z-gly-pro-**met**-his-OH
Z-gly-pro-**phe**-his-arg-OH	Z-gly-pro-**met**-his-arg-OH
Z-gly-pro-**phe**-his-arg-ser-OH	Z-gly-pro-**met**-his-arg-ser-OH

Figure Captions and Figures

Figure 1 : UV chromatograms for the Z-gly-pro-tyr peptide and the Z-gly-pro-ser peptide before and after incubation with the ZIP enzyme showing increased cleavage for the peptide containing tyrosine after proline in the peptide sequence.

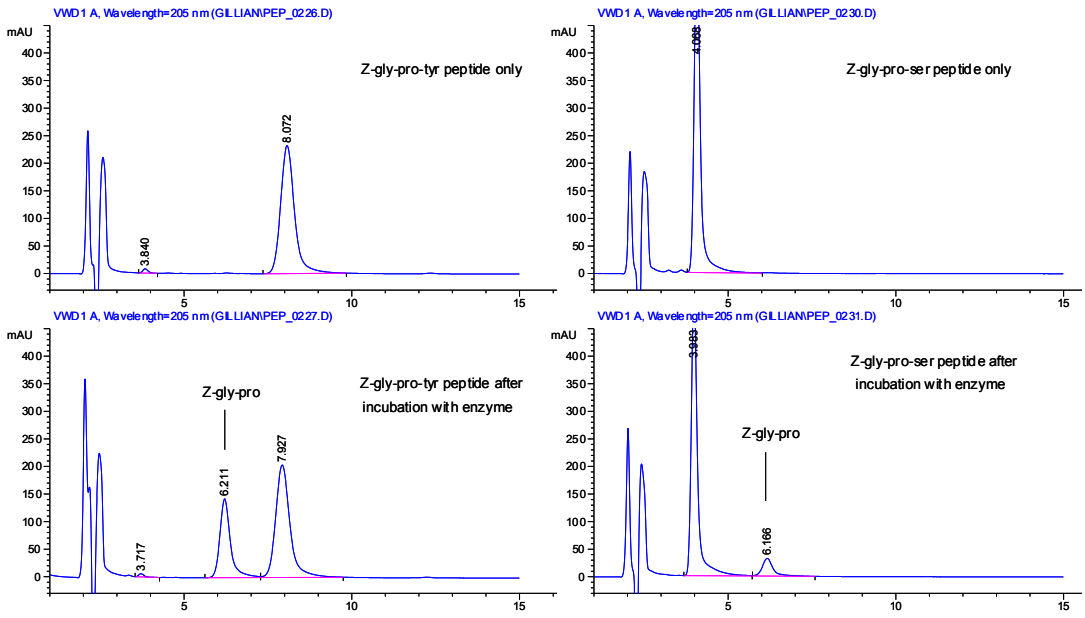


Figure 2 : UV chromatogram superimposed on the TIC (total ion current) trace for the Z-gly-pro-tyr peptide post-incubation sample and MS traces for both the parent peptide and the fragment Z-gly-pro.

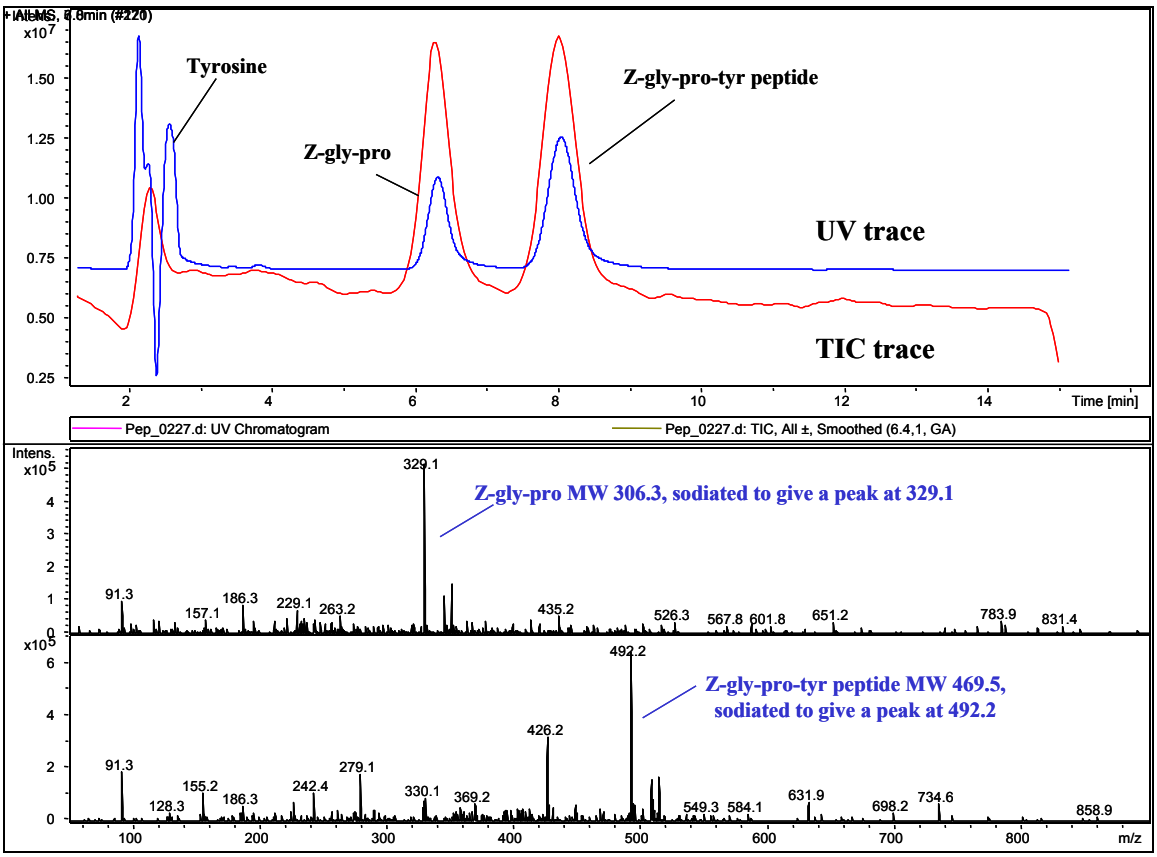


Figure 3 : UV chromatograms for four of the post-incubation peptide samples. In quantitative terms, 0.353mM, 0.197mM, 0.083mM and 0.015mM of Z-gly-pro were produced for peptides Z-gly-pro-tyr, Z-gly-pro-his, Z-gly-pro-ser and Z-gly-pro-glu respectively.

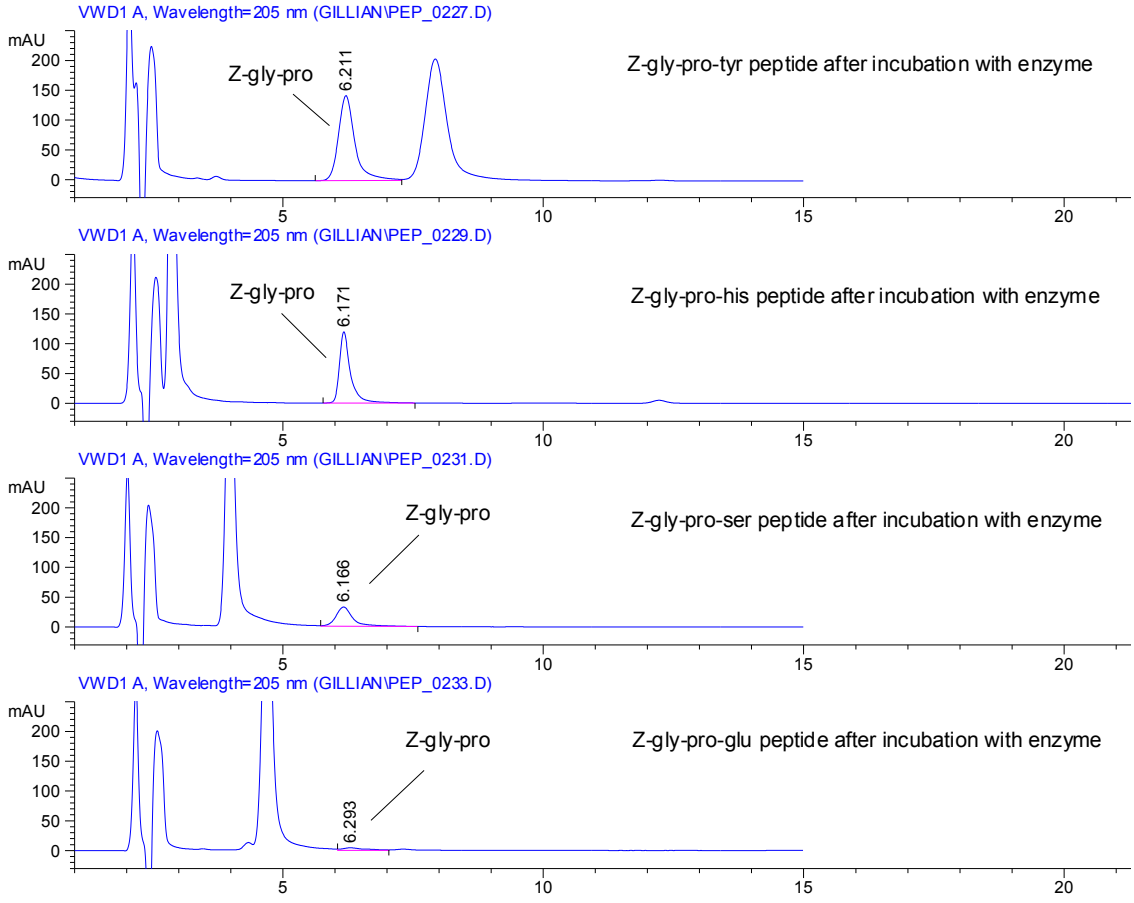


Figure 4 : UV chromatograms for the series of peptides based on Z-gly-pro-phe in an experiment to investigate the effect of chain length on the active site of the ZIP enzyme.

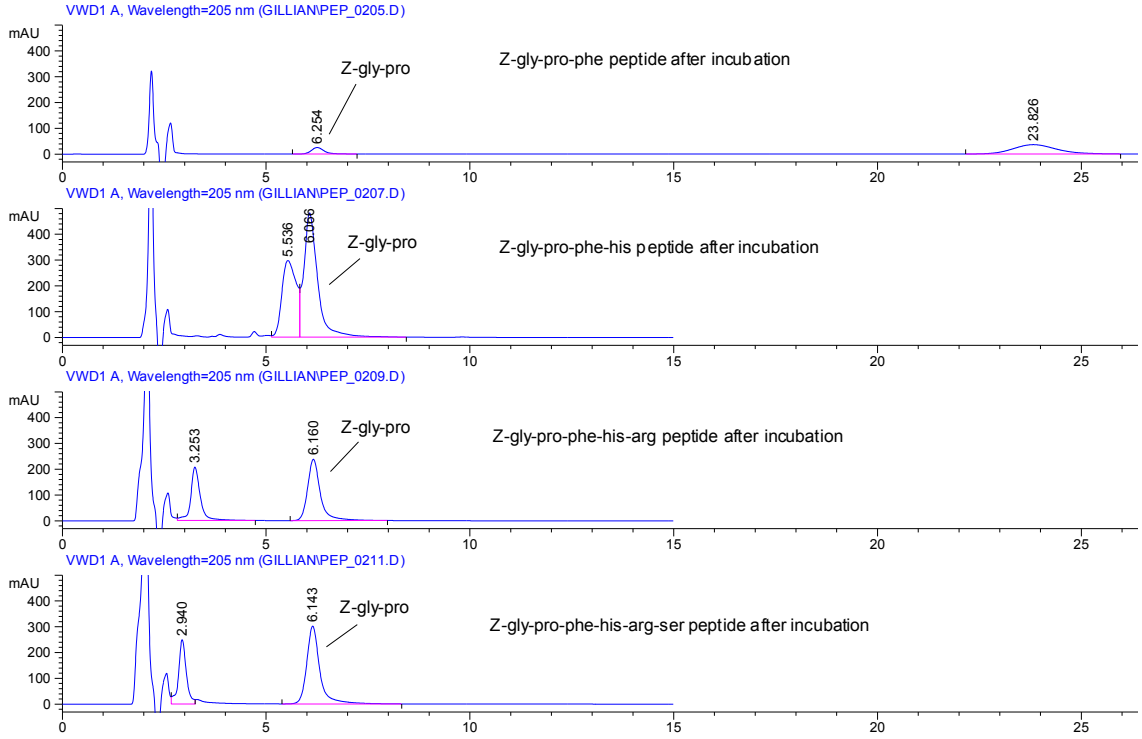
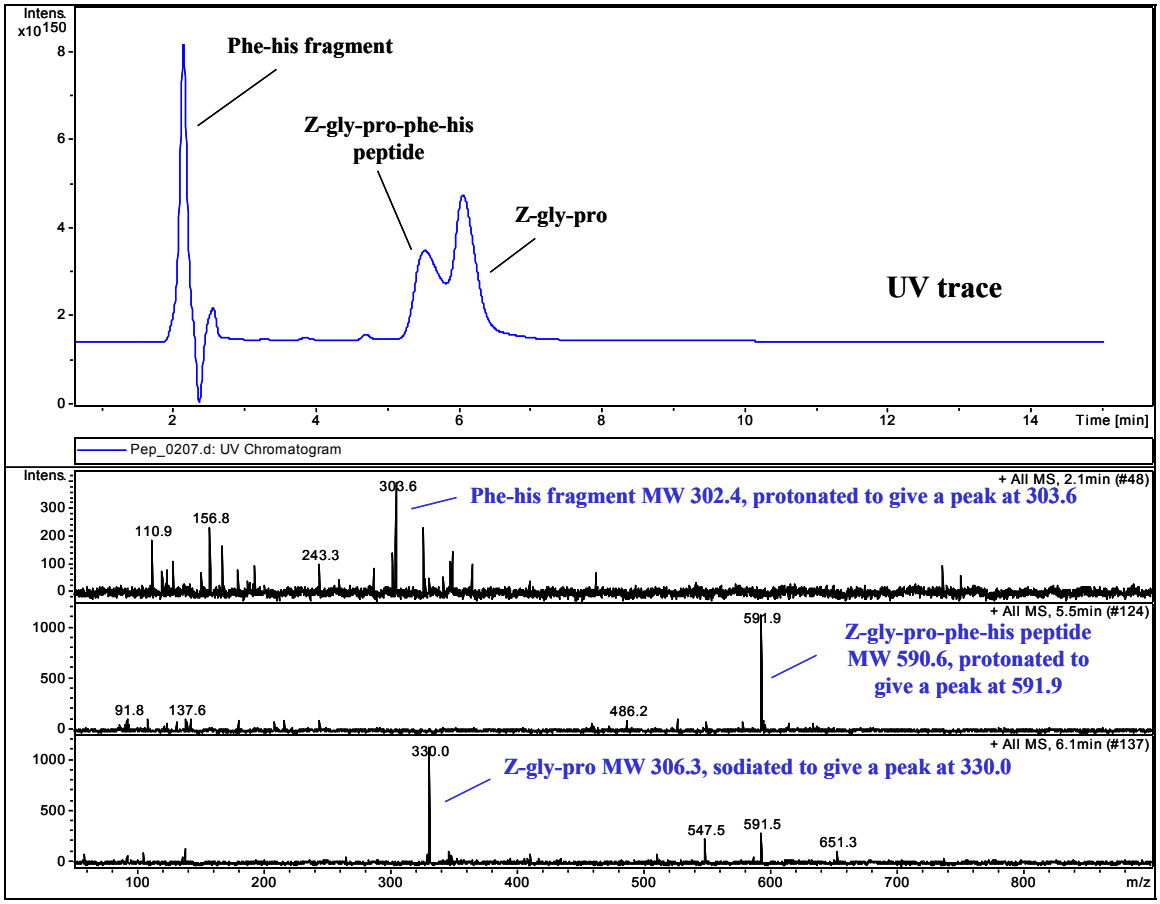


Figure 5 : UV chromatogram trace for the Z-gly-pro-phe-his peptide post-incubation sample and MS traces for the parent peptide and the two fragments Z-gly-pro and phe-his.



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