Peptide Synthesis by Recombinant *Fasciola hepatica* Cathepsin L1

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Abstract

Synthesis of the tripeptide Z-Phe-Arg-SerNH_2 has been accomplished by a recombinant cysteine protease, cathepsin L1 from liver fluke (*Fasciola hepatica*), using Z-Phe-Arg-OMe as acyl acceptor and SerNH_2 as nucleophile in 0.1 M ammonium acetate pH 9.0-12.5% v/v acetonitrile at 37^{o}C. LC-MS detection indicated tripeptide formation after 10 min, continuing up to 5.5 h. The ester Z-Phe-Arg-OMe was detected throughout the experiment but the hydrolysis product Z-Phe-Arg-OH appeared early and in quite large amounts. We believe that this is the first application of a parasite protease in enzymatic peptide synthesis.

Keywords: ***Fasciola hepatica*** cathepsin L1, recombinant, enzymatic peptide synthesis, cysteine proteinase

Abbreviations: LC-MS, liquid chromatography-mass spectrometry; *m/z*, mass-to-charge ratio; rFheCL1, recombinant *Fasciola hepatica* cathepsin L1; Z, benzoyloxycarbonyl.

Introduction

The helminth parasite *Fasciola hepatica* (liver fluke) causes disease in cattle and sheep and has recently emerged as an important pathogen of humans. The parasite secretes a protease, cathepsin L1 (EC 3.4.22.15), which plays an important role in many aspects of its pathogenicity (e.g. nutrient acquisition) by cleaving host proteins to absorbable peptides. Cathepsin L1 also cleaves matrix proteins such as fibronectin and collagen, thereby aiding parasite migration through host tissue (e.g. liver and intestine) and has been implicated in the inactivation of host immune defences by cleavage of immunoglobulins [1-4].

Cathepsin L1 is a lysosomal cysteine proteinase belonging to the papain superfamily. It has only endopeptidase activity and preferentially cleaves peptide bonds with hydrophobic amino acid residues in positions P_2 and P_3 [5]. The S_1' and S_2 binding sites are largely responsible for the enzyme's specificity, with the S_2 site being the major determinant. This subsite is a deep hydrophobic pocket, which accommodates large hydrophobic residues at the P_2 position [6,7]. Cathepsin L1 favours substrates with small amino acid side chains (Ala, Ser) or long but non-branched (Asn, Gln, Lys) at the P_1' position [8].

In recent years, proteases have been widely used to form peptide bonds. Enzymatic peptide synthesis offers an alternative to chemical methods, with numerous advantages: reactions take place under mild conditions and with high regiospecificity, side chain protection is unnecessary and no racemization occurs [9]. Compared with serine proteases, however, there have been few reports of the use of cysteine proteases in enzymatic peptide synthesis. Recombinant cathepsin L1 (rFheCL1) has been successfully expressed at high level in yeast systems, while cathepsin L1 extracted from liver flukes is notably stable [2]. We considered

that this stable, conveniently available recombinant cysteine protease might be an interesting candidate for peptide synthesis. Here we report the synthesis of the tripeptide Z-Phe-Arg-Ser-NH_2 by rFheCL1 under kinetic control. We believe this to be the first time that a recombinant parasite protease has been used in this way.

Materials & Methods

Materials - The synthetic peptide ester Z-Phe-Arg-OMe·HCl and nucleophile H-SerNH_2.HCl were obtained from Bachem (Bubendorf, Switzerland) and were used as supplied. Diaminoethanetetra-acetic acid (EDTA), formic acid and sodium acetate anhydrous were from Fisher Scientific. Acetonitrile and water (both CHROMASOLV® grade) were obtained from Riedel-de-Haen GmbH while DL-dithothreitol (DL-DTT) was from Sigma. Prof J.P. Dalton's Parasitology Research Group (Institute for the Biotechnology of Infectious Diseases, University of Technology, Sydney, Australia) generously supplied purified recombinant *Fasciola hepatica* Cathepsin L1 (rFheCL1; 0.5mg protein/ml) as a gift.

Activation of rFheCL1 – The enzyme was activated as required to 0.05mg rFheCL1/ml in a mixture comprising rFheCL1 (0.5mg/ml; 0.05ml), DTT (100mM; 0.04ml) and EDTA (500mM; 0.01ml) made up to total volume of 0.5ml with 0.1M sodium acetate, pH 5.0. The mixture was incubated at 37°C for 2 hr, then stored at -20°C until use.

Enzymatic Peptide Synthesis - Enzymatic synthesis of Z-Phe-Arg-SerNH_2 using Z-Phe-Arg-OMe HCl (ester) and Ser-NH_2 HCl (amine) was performed based on methods described by Stehle *et al*. [10] and Lozano *et al*. [11]. rFheCL1 was activated to a final concentration of 0.05mg/ml. Z-Phe-Arg-OMe was prepared as a 20mM stock solution in 100% (w/v) acetonitrile while the nucleophile, Ser-NH_2, was prepared to 50mM final concentration in 0.1M ammonium acetate, pH 9.0. Enzyme, nucleophile and ester were pre-incubated

separately in a waterbath at 37°C for 15 min prior to assay to ensure thermal equilibrium. In a 5ml volume test tube, 0.25ml Z-Phe-Arg-OMe and 0.4ml Ser-NH_2 were combined and the volume was adjusted to 2ml with 0.1M ammonium acetate, pH 9.0. Reaction was initiated by the addition of 0.25ml rFheCL1 (0.05mg/ml final conentration) and incubated at 37°C with stirring. Aliquots (0.2ml) were removed at intervals and mixed with 0.025ml acetonitrile:water:formic acid (50:25:25 v/v/v) to quench the reaction. Analysis was performed by liquid chromatography-mass spectrometry (LC-MS) on a Bruker Mass Spectrometer with electrospray ionisation and ion-trap detector linked to a Hewlett-Packard Esquire Liquid chromatograph 1100 with photodiode array detector. The LC isocratic method used a Zorbax SB-C18, 50 x 2.1 mm, 3.5µm narrow bore column with mobile phase 25:75:0.1 (v/v/v) acetonitrile:water:formic acid, flow rate 0.2ml/min. Monitoring was via a photodiode array detector at 200nm, injection volume 2µl.

Results

Concentrations of enzyme, ester and nucleophile rFheCL1-catalyzed synthesis of Z-Phe-Arg-Ser-NH_2 was performed as described above. Initially, enzyme, ester and nucleophile concentrations were 0.5mg/ml, 25mM and 50mM respectively, with an ester:nucleophile ratio of 1:2. Although formation of the desired tripeptide product was detected by mass spectrometry, it was not visible on the LC chromatographs, as a peak due to the ester was masking the tripeptide peak. Therefore, concentrations were reduced to 0.05mg/ml rFheCL1, 2.5mM Z-Phe-Arg-OMe and 10mM SerNH_2, giving a 1:4 ester:nucleophile ratio.

Tripeptide formation As shown in Figure 1, the tripeptide was formed within ten min of reaction commencement. The 10 min and 5.5 h traces clearly show two new peaks, eluting at 3.0 min (*m/z* 542.3 corresponding to the synthesised tripeptide Z-Phe-Arg-Ser-NH_2) and 3.4

min (*m/z* 455.2 ascribed to Z-Phe-Arg-NH_2). The major peak eluting at 4.1 min in all traces (*m/z* 456.2) is due to the hydrolysis product Z-Phe-Arg-OH.

Discussion

There are two strategies for enzymatic peptide synthesis: kinetically controlled and equilibrium controlled synthesis [9,12]. The kinetic method, using ester substrates, was preferred due to its lower enzyme requirements and shorter reaction times.

Specificity of the enzyme for amino acids at the P_1' position is an important and deciding factor when considering suitable nucleophiles for use in peptide synthesis. Literature indicates that cathepsin L has a preference for small amino acid side chains (Ala, Ser) or for long but non-branched (Asn, Gln, Lys) side chains at the P_1' position. It also indicates the order of preference among these residues at this site i.e. Ser > Ala > Lys > Asn > Gln [8]. Hence, serine amide was chosen as nucleophile for this synthesis.

The LC-MS traces in Figure 1 clearly show formation of a new peak eluting at 3 min (*m/z* 542.3), corresponding to the molecular weight of the desired tripeptide Z-Phe-Arg-Ser-NH_2. The new tripeptide could be detected in samples taken at 10 min and up to 5.5 h. We believe that the peak eluting at 3.4 min (*m/z* 455.2) represents Z-Phe-Arg-NH_2 formed by cleavage of the Ser N_α-C_α bond in the tripeptide product under the acid conditions used for reaction quenching and for liquid chromatography. The large peak at 4.1 min had a *m/z* value of 456.2, consistent with Z-Phe-Arg-OH. This may have arisen on storage of the ester substrate or during sample treatment. We performed peptide synthesis at pH 9.0 in order to maximize the concentration of the unionized –NH_2 form of the serine amide nucleophile. Use of more neutral pH values nearer for the synthetic reaction may reduce the incidence of non-enzymatic ester hydrolysis.

The mass spectrometric data obtained in this work confirmed for us the molecular weights of the separated reaction components detected by liquid chromatography. The extra breakdown product of the synthesised tripeptide with the *m/z* value of 455.2 was not expected and hence tandem mass spectrometry was not carried out during these experiments. However, further work on this project would include fragmentation MS experiments so as to elucidate structures of both predicted and unpredicted compounds. Neither the rate nor the total amount of product synthesised have been established in the present study. Nevertheless, the results obtained prove that rFheCL1 is capable of peptide synthesis. We have yet to ascertain the effects of reaction medium, pH and temperature, or of varying the concentrations of enzyme, substrate and nucleophile, on the synthetic reaction.

Synthesis in organic solvents prevents substrate ester hydrolysis as well as unwanted proteolysis [12]. Careful selection of the organic solvent for enzyme-catalysed peptide bond synthesis is essential, however, as the solvent affects not only the enzyme stability, but also solubility of the substrate and yield of peptide product [13]. Stability studies on rFheCL1 [14] demonstrated maximum activity in 50% (v/v) acetonitrile. The ester substrate is also soluble in this solvent; hence, this would be a good starting point for further investigation of rFheCL1-catalyzed peptide synthesis in organic solvents.

Investigation of the optimum pH for maximum synthetic activity would be instructive, as pH can influence kinetically controlled synthesis in two ways (one, binding of the ester substrate to the enzyme, leading to esterase activity and, two, deacylation of the acyl-enzyme by the nucleophile, leading to synthesis [11]).

Optimization of the reaction temperature would also be worthwhile. Kinetically controlled peptide synthesis has been performed at many temperatures ranging from frozen aqueous systems up to 40°C. Haensler *et al*. [15] demonstrated that α-chymotrypsin and papain were capable of kinetically controlled synthesis using unprotected amino acids as acyl acceptors in

a frozen aqueous system, with yields ranging from 43-95% depending on the acyl acceptor. Freezing is thought to increase the concentration of reactants in the unfrozen liquid phase that is in equilibrium with the ice crystals. Decreasing the temperature also reduces the extent of hydrolytic side reactions that take place at higher temperatures.

The concentrations of reactants greatly influence the rate of product formation. Decreasing the concentration of the reactants enhanced the resolution of the LC chromatographs; this could be further optimized. It would be interesting to establish the highest ester:nucleophile ratio that would still allow product formation and to determine the saturation level where maximum tripeptide formation is achieved. Here, peptide synthesis was obtained at a 1:4 ratio. Lozano *et al*. [11] used papain to catalyse the synthesis of a tripeptide using an ester:nucleophile ratio of 1:6. Elucidation of which nucleophile and acyl acceptor give the highest rate of tripeptide formation would also be interesting. Results could be compared with those of Ménard *et al*. [8], who concluded that cathepsin L favoured serine (amongst other amino acids) at the P_1' position.

The overall usefulness of rFheCL1 as a peptide ligase could be assessed in comparison with, e.g., papain and trypsin in model synthesis reactions under identical conditions. Both of these well-characterized proteinases have been used previously for peptide synthesis and share with rFheCL1 a preference for Arg residues in the P_1 position. Both papain and rFheCL1 are cysteine proteinases, affording further instructive comparisons.

Conclusion

Successful synthesis of the tripeptide Z-Phe-Arg-SerNH_2 has been achieved with recombinant rFheCL1, although the reaction needs further optimization. Review of the literature did not reveal any reports of peptide synthesis by parasite proteases to date, so the present findings appear to be novel.

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Legend to Figure

Figure 1. LC-MS traces of cathepsin L1-catalysed synthesis of Z-Phe-Arg-SerNH$_2$.

LC traces of samples taken at times zero, 10 min and 5.5 h are indicated on the main figure. The 10 min and 5.5 h traces clearly show two new peaks, eluting at 3.0 min (*m/z* 542.3, corresponding to the synthesised tripeptide Z-Phe-Arg-Ser-NH$_2$) and 3.4 min (*m/z* 455.2, ascribed to Z-Phe-Arg-NH$_2$). The major peak eluting at 4.1 min in all traces (*m/z* 456.2) is due to the hydrolysis product Z-Phe-Arg-OH. The late-eluting diffuse peak on all three traces has *m/z* value of 470, consistent with the Z-Phe-Arg-O-Me acyl acceptor. For full details, see materials and methods.

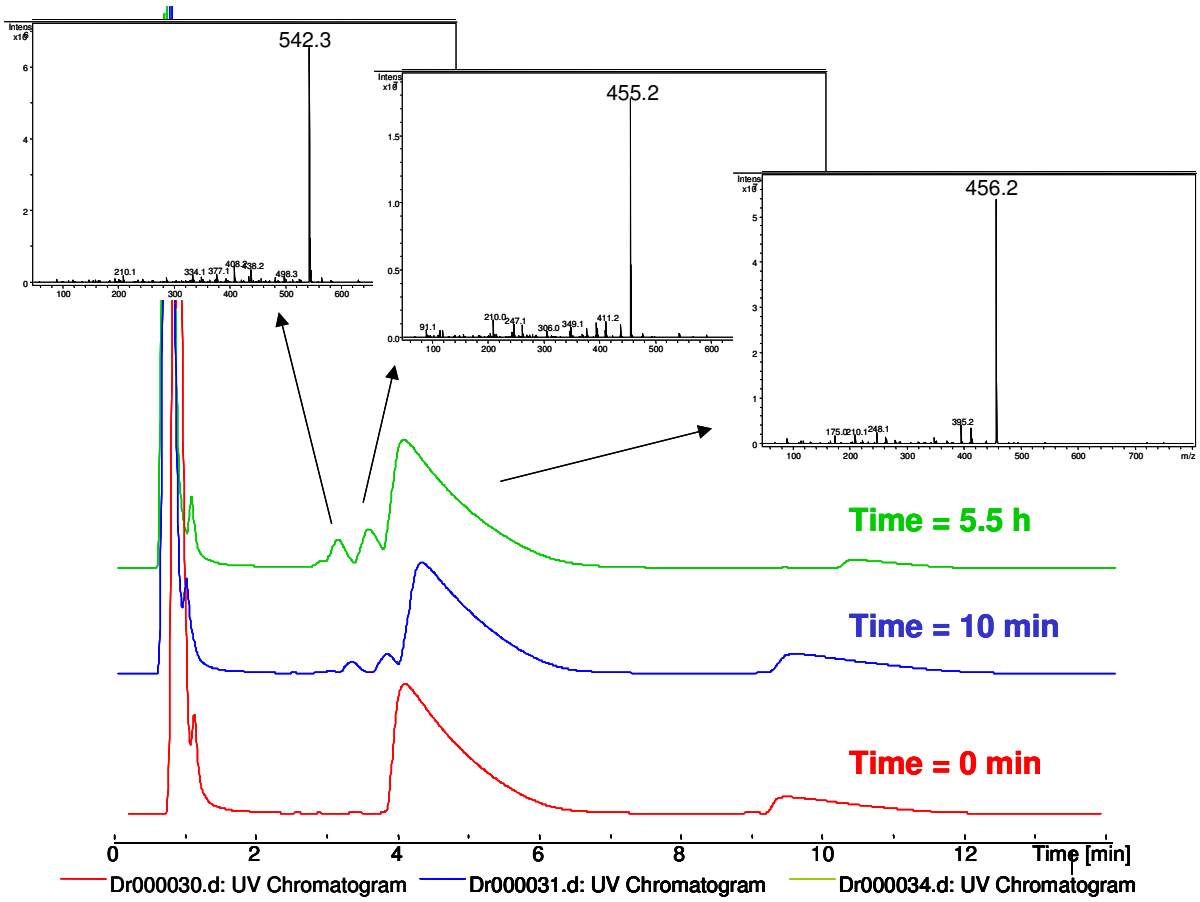
542.3
455.2
456.2
Time = 5.5 h
Time = 10 min
Time = 0 min
0
2
4
6
8
10
12
Time [min]
Dr000030.d: UV Chromatogram
Dr000031.d: UV Chromatogram
Dr000034.d: UV Chromatogram

Fig. 1.