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SEMISYNTHESIS III - THE HOMOSERINE ^{12,105} ANALOGUE OF HEN EGG-WHITE LYSOZYME.

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Abstract: The homoserine^{12,105} analogue of Hen egg-white lysozyme has been prepared by two routes. In the first approach a large excess of cyanogen bromide was used to cleave the native enzyme at methionines 12 and 105, the tertiary structure being maintained by the presence of the four disulphide bonds. Formation of the two new amide bonds was achieved using the activation of the homoserine lactone residues generated at positions 12 and 105; the reaction being most efficient when anhydrous DMSO was used as the solvent. An alternative approach using limited (two-fold excess) cyanogen bromide digestion gave the same homoserine analogue without chain fragmentation. A purified sample of the analogue showed no enzymic activity.

The preparation of a covalently linked 1-12/106-129 fragment of hen egg white lysozyme described in the previous paper[1] was obtained as a result of attempting to use methoxycarbonyl-sulphenyl chloride for sulphur protection during semisynthesis. As a result of the problems encountered with the methoxycarbonylsulphenyl group we revised our approach and examined the use of the sulphityl group for cysteine protection.

Previous studies[2] have shown that octa-sulphityl hen egg white lysozyme may readily be digested by cyanogen bromide, giving the anticipated 1-12, 13-105 and 106-129 fragments by cleavage at the methionines 12 and 105. Such a cleavage should, however, result in conversion of the methionine residues to C-terminal homoserine lactones. It was our intention to use this activated form of homoserine for coupling of protein fragments in a manner similar to that used by other workers.[3]

Cyanogen bromide fragmentation of the octa-sulphityl lysozyme, gave substantial quantities of each of the fragments enabling us to attempt coupling of the 1-12 and the 13-105 fragments with various amino components. Ultimately it was our objective to recombine the three fragments using synthetically modified 1-12 or 106-129 fragments, to obtain an analogue of lysozyme.

In the trial coupling experiments with a variety of amino acids, dipeptides and the 106-129 fragment of hen egg lysozyme no amide bond formation was observed. A wide variety of solvents and pH conditions were employed, including 0.1M sodium phosphate, 6M guanidine hydrochloride pH 8, and anhydrous DMSO. Relactonisation was also carried out according to the procedure of Wallace and Offord[4] and even under these conditions no reaction occurred at the homoserine lactone. Finally, conversion to the acyl azide prior to reaction was attempted as in some cases this has been a solution to the lack of reactivity of the homoserine lactone,[5] unfortunately again no reaction was observed.

As the activation provided by the homoserine lactone appeared to be insufficient to permit reaction, we therefore resorted to two different approaches which might result in the preparation of bis-homoserine 12, 105 lysozyme. In the first approach the procedure of Dykes *et al.*,[6] was used; this work reported that cyanogen bromide digested bovine pancreatic trypsin inhibitor (BPTI) spontaneously reformed intramolecular amide bonds on standing in neutral aqueous solution. In this case the homoserine lactone provided the activation for coupling, and the coupling was promoted by the chain cleaved materials being held together by an intramolecular disulphide bridge. This situation is similar to that found in cyanogen bromide digested lysozyme, in which the chain cleaved products are held together by the 6-127 and 30-115 disulphide bridges with the remaining 76-94 and 64-80 disulphide bridges within the 13-105 chain (see Figure 1). Earlier work in this area[7] had shown that hen egg white lysozyme could be cleaved by cyanogen bromide at methionines 12 and 105 and that the fragments 1-12, 13-105 and 106-129 were released after treatment with

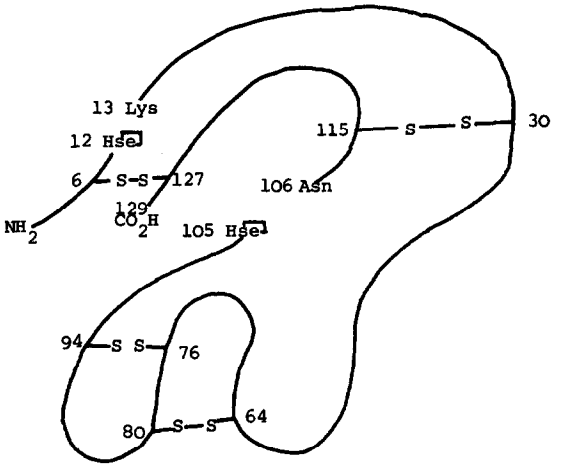

FIGURE 1 Simplified structure of hen egg white lysozyme after cyanogen bromide cleavage.

mercaptoethanol. Thus, it is possible that this approach would provide a means of preparing a bis-homoserine lysozyme analogue.

A sample of native hen egg white lysozyme was dissolved in 70% aqueous formic acid and digested with cyanogen bromide for 15 hours, the solvent was evaporated and the residue dissolved in distilled water then the solution was frozen and lyophilised. The product was divided into two portions (a) and (b); the first portion was suspended in DMSO/0.1M sodium phosphate buffer at pH 6.2 and left at 37°C for five days. The second portion (b) was dissolved in anhydrous DMSO but otherwise treated in the same way. These conditions should bring about the reformation of amide bonds in a manner similar to that used in the case of BPTI.[6]

A sample of the products resulting from both reactions was subjected to sulphitolysis[1] which should give a single product in the case of native hen egg white lysozyme and in the case of bis-homoserine analogue, but if the fragments had remained uncombined it would give the sulphityl derivative of the 1-12, 13-105 and 106-129 fragments. The possibility of reformation of only one of the amide bonds exists and further complicates the issue.

The products of sulphytilation were then applied to Sephadex G50 eluting with 70% aqueous formic acid. The gel filtration traces are shown in Figure 2 and from these results it can be

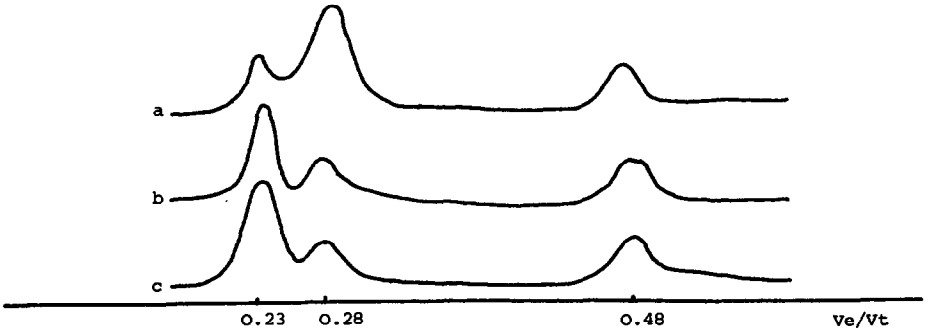

FIGURE 2 Gel filtration on Seaphadex G50 eluting with 70% aqueous formic acid following sulphitolysis of material from:- (a) recombination in DMSO/ 0.1M sodium phosphate pH 6.2, (b) recombination in anhydrous DMSO, (c) limited cyanogen bromide digestion. Monitored at 280 nm.

seen that several components were present. The peak at Ve/Vt 0.48 was identified by amino acid analysis as the 106-129 fragment and analysis of peaks eluting after this peak indicated that no

1-12 fragment was present. Thus, the material at Ve/Vt 0.28 was most probably the 1-105 fragment (as no 1-12 fragment was isolated), or possibly the 13-105 fragment. Some uncertainty exists over the identification of this peak as there is some overlap with the peak at Ve/Vt 0.23. The peak at Ve/Vt 0.23 eluted at a position which was identical to that of native hen egg white lysozyme, however, amino acid analysis indicated that the peak contained only approximately 4% of the methionine found in native lysozyme. At this stage we concluded that this material was the homoserine 12, 105 analogue of hen egg white lysozyme.

The relative size of the peak at 0.23 suggested that the amide bond reformation had proceeded more efficiently in anhydrous conditions than in 0.1M phosphate /DMSO.

Simultaneously with the above recombination experiments, another approach to the preparation of bis-homoserine 12, 105 lysozyme analogue was investigated. It is known that when only small excesses of cyanogen bromide over the methionine residues are used, the methionyl residues may be converted into homoseryl residues without peptide bond cleavage.[8,9]

A sample of hen egg white lysozyme was, therefore, treated with a two-fold excess of cyanogen bromide, and the reaction worked up and subjected twice more to treatment with cyanogen bromide under the same conditions. Samples were taken for amino acid analysis after each digest, and the methionine content fell from 70.5% after the first digestion to 3.5% after the final cyanogen bromide digestion. Part of the product was then subjected to sulphitolysis followed by gel filtration in the same way that had been used in the analysis of the recombination experiment discussed above. {The trace from this gel filtration (c) is shown in Figure 2 above.} In this case the largest peak was that at Ve/Vt 0.23, this peak contained only 3 - 4% free methionine by amino acid analysis, but again eluted at the position which would be anticipated for the octa-sulphityl 1-129 sequence. Analysis of the fractions after the last observed peak suggested that fragmentation had occurred at both methionine residues liberating the 1-12 and 13-105 fragments.

The desalted product from the recombination experiments in DMSO (b) and the limited cyanogen bromide digestion (c) were then compared with native hen egg white lysozyme by electrophoresis on cellulose acetate at pH 7 in 8M urea 0.1M phosphate buffer. Material from (b) had a mobility of 0.86 relative to that of lysozyme (-1.0) and material from c a mobility of -0.81 , thus both were virtually identical. As from electrophoresis there is a significant difference in mobility from native hen egg white lysozyme, further purification of the material from recombination (b) was attempted by further gel filtration and ion exchange chromatography using Sephadex CM50 running a gradient from 8M urea to 8M urea being 0.5M in sodium chloride.

Purification of the product from experiment (b) was achieved and a single peak was obtained from ion exchange chromatography as shown in Figure 3. Figure 3 also shows theposition of elution of native hen egg white lysozyme and of a 1:1 mixture of hen egg white lysozyme and the product of experiment (b). From these experiments we concluded that the homoserine 12, 105 analogue of lysozyme had been prepared. This was further corroborated by the amino acid analysis of the material isolated from ion exchange chromatography Trp(6) Nd Lys(6) 5.85 His(1) 0.85 Arg(11) 10.68 Asp(21) 20.30 Thr(7) 6.64 Ser(10) 8.77 Pro(2) 2.19{Glu(5)+Hse(2)} 6.16 Gly(12) 12.22 Ala(12) 12.17 ½Cys(8) - Val(6) 5.58 Met(2) 0.07 Ile(6) 5.79 Leu(8) 8.24 Tyr(3) 3.03 Phe(3) 2.98. The almost total absence of methionine and the high glutamic acid value indicate the presence of homoserine as these two residues coelute on amino acid analyser. Furthermore, it should be noted that the presence of two homoserine residues in the protein would not give rise to two homoserine

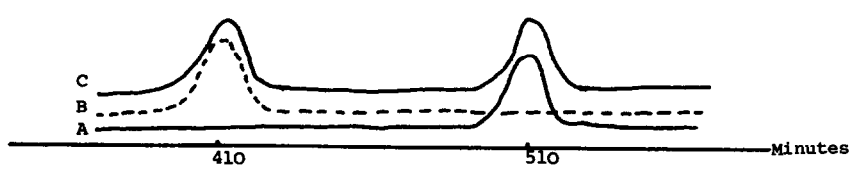

FIGURE 3 Ion exchange chromatography on Sephadex CM50 eluting on a gradient from 8M urea to 8M urea/0.5M NaCl. A - material isolated from the peak with Ve/Vt 0.23 from (b) above (see Figure 2); B - Native hen egg white lysozyme; C - 50/50 mixture of materials in A and B.

residues in the hydrolysate as homoserine is slowly degraded under acid hydrolysis similarly to serine.

The UV spectrum of the homoserine 12, 105 analogue was virtually identical to that of the native enzyme hence suggesting that the tryptophan residues were not affected by the conditions employed in the preparation of the analogue. In contrast cyanogen bromide treated hen egg white lysozyme with intact disulphide bonds without reformed amind bonds at homoserine 12 and 105 showed a slight red shift in the UV spectrum.[7]

The material from the ion exchange chromatography was exhaustively dialysed, lyophilised and assayed for lysozyme activity using the assay[10] based on the use of a bacterial cell wall preparation. We found that the homoserine 12, 105 analogue was completely inactive, this was slightly surprising as in many other cases homoserine containing enzyme analogues retain most of the activity of the native enzyme.[3] Interestingly native hen egg white lysozyme which had been digested with cyanogen bromide[7] having the disulphide linkages intact and not having reformed amide bonds at residues 12-13 and 105-106 was also found to have little activity; in this case 0.5% of the activity of native hen egg white lysozyme was retained. It is impossible to be sure of the reason for this lack of activity as it is known that neither of the methionine residues are directly involved in the active sites of the enzyme.[11] However, the asparagine residue at position 106 is involved in a hydrogen bonding interaction with the substrate and thus if the adjacent methionine residue were replaced by homoserine then a further hydrogen bonding site is introduced close to residue 106 and this may disrupt this area of the active site.

We were, therefore, satisfied that the homoserine 12, 105 analogue of lysozyme had been prepared by both a recombination method involving the retention of disulphide bonds, experiment (b), and by limited cyanogen bromide digestion, (experiment c).

EXPERIMENTAL

Materials and General Techniques.

Materials were purchased from the sources indicated in the preceeding papers.[1,2] On all occasions Visking 18/32 tubing was used and urea was freshly dionised. Spectra and amino-acid analyses were recorded using the instruments stated previously.[1]

Preparation of $Hse^{12,105}$ - Lysozyme by spontaneous amide bond formation following cyanogen bromide digestion of native hen egg white lysozyme.

Hen egg white lysozyme (200 mg, 14 μM) was dissolved in 70% aqueous formic acid (20 ml) and cyanogen bromide (74mg, 0.7 mM) added. The solution was stirred at room temperature for 15 hours, then evaporated *in vacuo* and the residue dissolved in distilled water (20 ml). This solution was frozen and lyophilised to give a residue (200 mg) which was divided into two equal portions, prior to further reaction.

(a) The material (100 mg) was suspended in DMSO/0.1M sodium phosphate, pH 6.2 (40 ml) and stirred for five days at 37°. The suspension was transferred to Visking tubing and dialysed against distilled water. The contents of the dialysis sac was then frozen and lyophilised to give a residue (60 mg). A portion (15 mg) of this material was subjected to sulphitolysis using the procedure described previously.[2] After dialysis the product was subjected to gel filtration on Sephadex G50 eluting with 70% aqueous formic acid. The elution profile shown in Figure 2 was obtained with major peaks eluting at Ve/Vt 0.23, 0.28 and 0.48. Amino acid analyses: Ve/Vt 0.23 (sulphitylated $Hse^{12,105}$ lysozyme): Trp(6) Nd, Lys(6) 6.38, His(1) 1.10, Arg(11) 10.40, Asp(21) 21.91, Thr(7) 6.58, Ser(10) 8.25, Glu (+Hse){5(+2)} 6.03, Gly(12) 12.21, Ala(12) 12.00, ½Cys Nd, Val(6) 6.24, Met(0) 0.08, Ile(6) 5.82, Leu(8) 8.18, Tyr(3) 2.91, Phe(3) 2.63; Ve/Vt 0.28 (sulphitylated 1-105 but is not conclusive): Trp(3) Nd, Lys(5) 4.25, His(1) 0.95, Arg(7) 7.12, Asp(18) 16.78, Thr(6) 5.76, Ser(10) 7.46, Pro(2) 1.40, Glu(4) 4.00, Gly(10) 10.10, Ala(9) 7.81, ½Cys(6) Nd, Val(4) 4.00, Met(0) 0.38, Ile(5) 5.63, Leu(7) 6.88, Tyr(3) 2.52, Phe(3) 2.27; Ve/Vt 0.48 (sulphitylated 106-129): Trp(3) Nd, Lys(1) 1.05, Arg(4) 3.68, Asp(3) 2.85, Thr(1) 0.71, Ser(0) trace,Glu(1) 1.20, Gly(2) 2.02, Ala(3) 3.11, Val(2) 1.91, Ile(1) 0.94, Leu(1) 1.16.

(b) The lyophilised product (100 mg) was dissolved in anhydrous DMSO (20 ml) and left at 37° for 5 days. The solvent was evaporated *in vacuo* and the residue dissolved in distilled water (40 ml), prior to dialysis in Visking tubing against distilled water. The contents of the dialysis sac were frozen and lyophilised giving a residue (73 mg). A sample (15 mg) of this

material was subjected to sulphitolysis[2] and following dialysis of the resulting solution the product was subjected to gel filtration on Sephadex G50 eluting with 70% aqueous formic acid. The elution profile shown in Figure 2 was obtained, major peaks eluted at Ve/Vt 0.22, 0.27 and 0.48. Amino acid analyses: Ve/Vt 0.22 (sulphitylated Hse[12,105] lysozyme), Trp(6) Nd, Lys(6) 6.66, His(1) 1.28, Arg(11) 10.74, Asp(21) 21.06, Thr(7) 5.16, Ser(10) 4.76, Glu(+Hse) {5(+2)} 5.46, Pro(2) 2.36, Gly(12) 12.60, Ala(12) 10.02, ½Cys Nd, Val(6) 5.34, Met(0) 0.03, Ile(6) 6.00, Leu(8) 8.56, Tyr(3) 3.18, Phe(3) 2.58; Ve/Vt 0.27 (suggests sulphitylated 1-105 but not conclusive): Trp(3) Nd, Lys(5) 4.55, His(1) 0.90, Arg(7) 7.75, Asp(18) 18.62, Thr(6) 5.89, Ser(10) 7.78, Pro(2) Nd, Glu(4) 4.18, Gly(10) 10.10, Ala(9) 8.91, ½Cys(6) Nd, Val(4) 4.00, Met(0) 0.0, Ile(5) 5.47, Leu(7) 6.58, Tyr(3) 2.36, Phe(3) 2.44; Ve/Vt 0.48 (sulphitylated 106-129) Trp(3) Nd, Lys(1) 0.95, Arg(4) 3.85, Asp(3) 3.10, Thr(1) 0.80, Glu(1) 1.25, Gly(2) 2.05, Ala(3) 3.15, Val(2) 2.05, Ile(1) 1.00, Leu(1) 1.20.

Ion exchange purification of Hse[12,105] lysozyme from procedure (b) above.

A portion (50 mg) of the material from (b) above (recombination in anhydrous DMSO) was subjected to gel filtration on Sephadex G50 eluting with 70% aqueous formic acid. The major peak had Ve/Vt 0.23 and showed some tailing. Fractions from the central portion of this peak were pooled and recromatographed using the same system, once again the fractions comprising the centre of the peak were pooled, frozen and lyophilised. Amino acid analysis of the reisdue Trp(6) Nd, Lys(6) 6.60, His(1) 1.17, Arg(12) 12.22, Asp(21) 19.24, Thr(7) 6.11, Ser(10) 7.55, Glu (+Hse) {5(+ 2)} 5.90, Pro(2) 2.32, Gly(12) 11.60, Ala(12) 12.39, ½Cys(8) Nd, Val(6) 5.67, Ile(6) 5.38, Leu(8) 7.84, Tyr(3) 2.85, Phe(3) 2.91. This material was applied to Sephadex CM50 (column 1.5 cm x 18 cm); initial eluant 8M urea, final eluant 8M urea/0.5M NaCl mixing in a 250 ml mixing vessel, flow rate 32 ml h^{-1}, fractions collected at 10 min. intervals as detected by absorption at 280 nm. A symmetrical peak eluted at 410 min, (native hen egg white lysozyme elutes at 510 minutes), the central fractions of this peak were pooled. Exhaustive dialysis against distilled water was carried out to remove urea and NaCl and the final product obtained by lyophilisation of the contents of the dialysis sac. The amino acid analysis is given in the discussion above.

Preparation of Hse[12,105] - lysozyme by limited cyanogen bromide digestion.

Hen egg white lysozyme (200 mg, 14 μM) was dissolved in 70% aqueous formic acid (20 ml), cyanogen bromide (3 mg, 28 μM) added and the solution stirred for 24 hours at room temperature. A second portion of cyanogen bromide (3 mg, 28 μM) was added and the solution stirred for a further 24 hours at room temperature. Distilled water (200 ml) was added and after the solvent volume had been reduced to ca. 20 ml the remaining solution was frozen and lyophilised. A sample (50 mg) of this material was subjected to sulphitolysis[2] and processed in the same manner as described in the previous experiment. The elution profile is shown in Figure 2(c), major peaks eluted at Ve/Vt 0.24, 0.28 and 0.47. Amino acid analyses Ve/Vt 0.24 (sulphitylated Hse[12,105] lysozyme): Trp(6) Nd, Lys(6) 5.67, His(1) 1.16, Arg(11) 9.17, Asp(21) 23.20, Thr(7) 6.61, Ser(10) 9.55, Glu(5) 4.88, Pro(2) 1.67, Gly(12) 11.88, Ala(12) 9.16, ½Cys(8) Nd, Val(6) 4.45, Met (0) 0.04, Ile(6) 6.00, Leu(8) 8.00, Tyr(3) 3.61, Phe(3) 2.61; Ve/Vt 0.28 (sulphitylated 13-105) Trp(3) Nd, Lys(4) 3.65, His(1) 0.83, Arg(6) 5.50, Asp(18) 15.90, Thr(6) 4.91, Ser(10) 6.70, Pro(2) 2.09, Glu(+Hse{3(+1)} 3.64, Gly(9) 8.35, Ala(6) 6.64, ½Cys(5) Nd, Val(3) 2.98, Met(0) 0.16, Ile(5) 4.27, Leu(6) 5.58, Tyr(3) 1.61, Phe(2) 1.92; Ve/Vt 0.47 (sulphitylated 106-129) Trp(3) Nd, Lys(1) 1.12, Arg(4) 3.61, Asp(3) 2.92, Thr(1) 0.95, Glu(1) 1.13, Gly(2) 2.00, Ala(3) 3.08, ½Cys(2) Nd, Val(2) 1.91, Ile(1) 0.89, Leu(1) 1.12; (fractions eluting after peak at Ve/Vt 0.47 pooled, (sulphitylated 1-12) Lys(1) 1.09, Arg(1) 0.99, Glu(+Hse){1(+1)} 1.22, Gly(1) 1.10, Ala(3) 2.89, ½Cys(1) Nd, Val(1), 0.97, Met(0) 0.09, Leu(1) 0.98, Phe(1) 0.94.

Assay of lysozyme activity.[10]

A suspension of dried cells of micrococcus lysodeikticus in 0.1M phosphate buffer at pH 7 and a calibration standard of hen egg white lysozyme (0.05 mg ml^{-1}) were prepared. An accurate determination of the enzyme concentration was made by measuring the OD_{280} taking the extinction coefficient to be 35712. The solution to be assayed (0.1 ml) and the suspension of dried cells (2.9 ml) were mixed and the decrease in turbidity at 450 nm measured over 2 minutes recording at 15 second intervals. The definition[10] "One unit of activity is equal to a decrease in absorbance

of 0.001 min^{-1} at pH 7.0", was used to calculate the enzymic activity.

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