THE AMINO-TERMINAL SEQUENCE OF SILK FIBROIN PEPTIDE Cp - A REINVESTIGATION

Daniel J. Strydom[a], Thomas Haylett[a] and Robin H. Stead[b]

[a]National Chemical Research Laboratory, Council for Scientific and Industrial Research, P.O. Box 395, Pretoria, 0001,

and

[b]Western Province Blood Transfusion Service, P.O. Box 3788, Cape Town, 8000, South Africa.

Received October 31, 1977

Summary: A new amino-acid sequence is proposed for silk fibroin peptide Cp, after automatic Edman degradation studies. The proposed sequence is: Gly-Ala-Gly-Ala-Gly-Ser-Gly-Ala-Ala-Gly-$(Ser\text{-}Gly\text{-}(Ala\text{-}Gly)_n)_8$ Tyr, where n is usually 2.

The amino-terminal sequence of peptide Cp from silk fibroin was proposed by Lucas et al. (1) to be: Gly-Ala-Gly-Ala-Gly-$(Ser\text{-}Gly\text{-}(Ala\text{-}Gly)_n)_8$-Ser-Gly-Ala-Ala-Gly-Tyr, where n is usually 2. It is pertinent to stress that despite the creeping tendency in the literature to call this sequence "established", Lucas et al. (1) specifically stated that this sequence is "probable" and that the position of the Ser-Gly-Ala-Ala-Gly part of the sequence is a "tentative suggestion".

During studies by Stead and P.L. Mustart (to be published) on the incorporation of radioactively labelled alanine and glycine into silk fibroin, we had occasion to investigate the amino-terminal sequence of peptide Cp. In addition, synthesis of radioactive silk fibroin in Xenopus oocytes after injection of messenger RNA provided an opportunity to confirm the chemical sequence of Cp by estimating the release of radioactivity during sequential cleavage.

MATERIALS AND METHODS

Lithium thiocyanate, used to solubilise fibroin preparations, was prepared by

ISSN 0006-291X

mixing equimolar quantities of LiOH and NH_4SCN with heating in a final volume of 125 ml per mole of LiSCN. Residual ammonia was removed and the solution concentrated by vacuum evaporation, recrystallised from water, the crystals redissolved in a minimum volume of water and standardised with $AgNO_3$ to 70% (w/v) LiSCN.

Frozen middle silk glands of Bombyx mori were thawed to 20° and incubated with hyaluronidase (1 mg/ml) and collagenase (0.5 mg/ml) in saline (0.15M) sodium citrate (0.015M) solution (10 ml enzyme solution added to 8 g of glands) for 1 h. They were rinsed three times in distilled water (by soaking for 15 min and decanting) and dissolved in 70% LiSCN (2 ml/g glands) with gentle stirring. Insoluble debris was removed by centrifugation, and the solution made 0.1M with respect to mercaptoethanol and 0.1% (w/v) with respect to EDTA. After 30 min distilled water was added to give a tenfold dilution, and LiSCN was removed by dialysis against 0.01M mercaptoethanol at 4° until no further SCN^- was detectable with 1% $FeCl_3$. The concentration of fibroin [$E^{1\%}_{1\ cm}$ = 11.1 at 280 nm (2)] was about 2 mg/ml.

To prepare Cp, 1M K_2HPO4 (1/10 volume) and chymotrypsin (1 mg/ml) were added to dialysed soluble fibroin. Following incubation at 37° for 4 h, Cp was recovered as a crystalline precipitate which was washed successively in distilled water, 0.1N HCl, ethanol and ether.

Oocytes were incubated in ^{14}C glycine and ^{3}H alanine following injection of silk fibroin messenger RNA. Labelled peptide Cp was extracted as described above and purified by chromatography on Biogel P-10 using 4M guanidine-HCl as eluant, to yield the fraction Cpc. Sequence analysis for release of radioactivity was performed on Cpc, since Cp was found to contain non-specifically bound ^{3}H-ala.

A Beckman 890B sequencer was employed for sequence analysis, and use was made of dimethylbenzylamine as buffer (3). As was expected with the large number of glycyl residues, considerable overlap problems were experienced and we repeated the analysis under a number of varying conditions. Four different programmes were used:

1. The single cleavage procedure (Beckman Program Protein DMBA, 102473 Mod. 122974) base on the programme of Hermodson *et al.* (3),

2. an extra cleavage step introduced,

3. the coupling step repeated before doing a single cleavage and

4. the coupling step repeated, and a double cleavage done.

Thiazolinones were converted to phenylthiohydantoins by heat conversion (4) at 80° for 30 min but with 1 µl 10% trifluoroacetic acid added to ensure the presence of acid which appeared to be crucial for the conversion step. The phenylthiohydantoins were identified by gas chromatography according to Pisano and Bronzert (5) on SP 400 as stationary phase.
The labelled peptide was degraded with programme 4 since this gave the best results. The converted phenylthiohydantoins (in methanol) were checked by thin layer chromatography with chloroform-ethylacetate (9:1) (6).
The remainder of each fraction was solubilised in 5 ml Instagel (Packard Instruments) in polyethylene vials, and counted in a Packard Model 2650 spectrometer. Due to the small amount of radioactivity released at each cleavage cycle, samples were counted for $1x10^3$ min, and the automatic

Table I. Amino acid composition of fibroin peptide Cp

Amino acid	Residues/mole*
Lysine	0.13
Histidine	0.04
Arginine	0.11
Aspartic acid	0.34
Threonine	0.22
Serine	9.08 (9)
Glutamic acid	0.26
Proline	0
Glycine	29.12 (29)
Alanine	19.95 (20)
Valine	0.39
Methionine	0
Isoleucine	0.08
Leucine	0
Tyrosine	0.85 (1)
Phenylalanine	0.08

* Calculated to yield 29 residues of Ser+Ala.

discrimination programme was checked with standards at low activity. Under the conditions used, the mean coefficient of variation associated with the $^{14}C/^{3}H$ ratio was 5%, and spillover of ^{3}H in the ^{14}C channel after discrimination was less than 0.2%. The amino-acid composition of the peptide was determined on an acid hydrolysate.

RESULTS AND DISCUSSION

The results of amino acid analysis of peptide Cp are given in Table 1 and agree with the composition of the sequence proposed by Lucas et al. (1).

Table II. Gas chromatographic analysis of phenylthiohydantoin derivatives obtained by sequenator degradation of fibroin peptide Cp.

Cycle	Programme 1			2			3			4			Results
	Ala	Gly	Ser	Ala	Gly	Ser	Ala	Gly	Ser	Ala	Gly	Ser	
1	0	36	4	0	44		0	24		0	59		Gly
2	44	10	5	100	23		50	6		122	8		Ala
3	19	23	3	29	35		22	20		36	46		Gly
4	34	16	5	48	21		50	9		86	17		Ala
5	23	19	5	31	25	5	38	21	1	34	23	1	Gly
6	16	19	7	18	19	6	15	12	4	20	14.5	3	Ser
7	9	19	8	13	25		11	17		19	25	3	Gly
8	15	17	7	21	21		28	13.5		41	17		Ala
9	20	13	5				38	11	3	48	11		Ala
10	26	14	8				33	10	4	35	11		
11							19.5	9		30	14		
12							25	11		29	15		
13										26	12		
14										21	9		

Peak heights are given in arbitrary units based on the same scale attenuation.

In Table II we present the quantitative results of four degradation experiments. The figures are in arbitrary units (peak heights on the chart paper) but are all based on the same scale attenuation of the gas chromatograph. The yield of alanine at cycle 2 (programme 4) was 80% with a repetitive yield of 85%.

This yields the following amino-terminal sequence for fibroin peptide Cp:

Gly-Ala-Gly-Ala-Gly-Ser-Gly-Ala-Ala .....

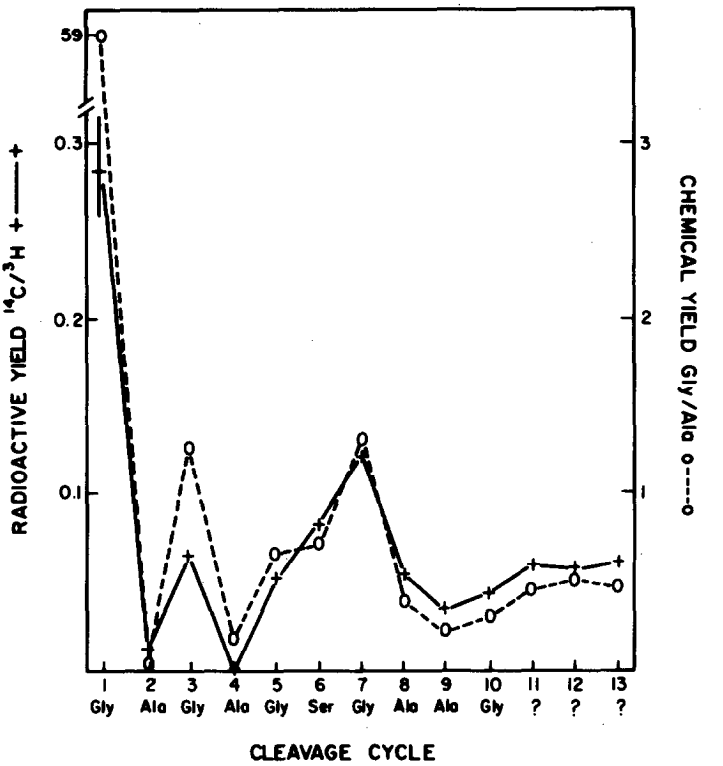

Figure 1: Radioactive and chemical yield following sequential degradation of Cp from silk fibroin messenger - injected oocytes

One hundred Xenopus oocytes injected with silk fibroin messenger RNA were incubated in 1-^{14}C-glycine and L-(2,3-^{3}H) alanine, and radioactive Cp extracted under conditions described in the text. Chemical yields were obtained from the data of experiment 4, Table II, while radioactive yields were derived from an identical automatic cleavage of radioactive Cp purified by Biogel P-10 chromatography.

When the yield of ^{14}C and ^{3}H is compared with the chemical yields (Fig. 1) the chemical sequence is corroborated. Serine in position 6 yields ^{14}C as thought it were glycine, since the α-carbon of serine, derived metabolically from glycine, is retained in the dehydroalanyl derivative obtained from the sequenator. The chemical yield gives no change in the ratio at position 6, and the "cross-over" effect could be taken as diagnostic for serine.

The alanyl-alanyl sequence found at position 8-9 in this peptide at last

provides definite proof that the segment of peptide Cp which contains this rare sequence is near the amino terminus and not the carboxyl terminus of peptide Cp.

With this information, and additionally the indications that positions 10 to 12 were labelled with ^{14}C (indicative of serine and glycine), the following sequence is now suggested for silk fibroin peptide Cp: Gly-Ala-Gly-Ala-Gly-Ser-Gly-Ala-Ala-Gly-$(Ser\text{-}Gly\text{-}(Ala\text{-}Gly)_n)_8$-Tyr, where n is usually 2. This is consistent with the data derived from the sequencer runs (both chemical and radioactivity measurements) and from amino acid analyses of the whole peptide (this paper and (1)). It is also consistent with the results of sequencing studies by Lucas et al. (1) and Zahn et al. (7). This newly proposed structure does not affect proposals concerning the possible origin of periodic proteins (8) nor is it inconsistent with data of Suzuki and Brown (9) on fragments produced by ribonuclease T_1 digestion of silk fibroin messenger RNA, as both these studies are based on the hexapeptide repeating unit. The messenger RNA for this part of fibroin will, however, now have the predicted major sequence: GGX-GCU-GGX-GCU-GGX-UCA-GGX-GCU-GCU-GGX-$[UCA\text{-}GGX\text{-}(GCU\text{-}GGX)_2]_8$-$UA^U_C$, (where X=U or A), from the results of Suzuki and Brown (9).

ACKNOWLEDGEMENT

The technical assistance of Mrs. A. Ruelle is gratefully acknowledged.

REFERENCES

1. Lucas, F., Shaw, J.T.B., and Smith, S.G. (1957) Biochem. J., 66, 468-479.
2. Tashiro, Y., Otsuki, E. and Shimadzu, T. (1972) Biochim. Biophys. Acta, 257, 198-209.
3. Hermodson, M.A., Ericsson, L.H., Titani, K., Neurath, H., and Walsh, K.A. (1972), Biochemistry 11, 4493-4502.

4. Guyer, R.L., and Todd, C.W. (1975) Anal. Biochem., 66, 400-404.
5. Pisano, J.J., and Bronzert, T.J. (1969) J. Biol. Chem. 244, 5597-5607.
6. Cherbuliez, E. Baehler, B.R., Marzaleck, J., Sussman, A.R., and Rabinowitz, J. (1963) Helv. Chim. Acta, 46, 2446-2452.
7. Zahn, H., Schade, W., and Ziegler, K. (1967) Biochem. J., 104, 1019-1025.
8. Ycas, M. (1972) J. Mol. Evol. 2, 17-27.
9. Suzuki, Y., and Brown, D.D. (1972) J. Mol. Biol. 63, 409-429.