POST-MORTEM PROTEOLYTIC PROCESSES IN LIVER TISSUE

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In the course of post-mortem proteolysis, liver proteins are degraded to amino acids and oligopeptides without any significant cumulation of low molecular weight proteins and polypeptides in the tissue. The concentration of peptides during the proteolysis is low; it is relatively highest in the middle phase of the post-mortem process. The concentration of free amino acids in the liver tissue increases approximately 3·5-times during the 24 h post-mortem period at 37°C. A part of the soluble proteins which have been degraded in the course of the post-mortem proteolysis is replaced as a result of solubilization of the originally insoluble proteins.

The activity of tissue proteinases is well controlled under physiological conditions since a loss of the equilibrium in this system could have catastrophic consequences for the organism. The factors which affect the activity of proteinases under both physiological and post-mortem conditions involve changes in pH, in the concentration of various ions, in the ratio of activities of various proteinases, in the concentration of proteinase inhibitors, in the structure and properties of proteins, and in the concentration of various products of enzymic reactions.

In this study we have made an effort to describe the course of post-mortem proteolysis in the liver tissue by studying the changes in protein fractions and proteolytic fragments.

EXPERIMENTAL

Chemicals: Sephadex G-100 and G-25 were preparations of Pharmacia, Uppsala, egg albumin was from Biochemical Corporation, bovine albumin from Koch Light, chymotrypsin and alcohol dehydrogenase from Reanal. The A-chain of oxidized insulin was kindly supplied by Dr H. Keilová, Institute of Organic Chemistry and Biochemistry, Prague. The remaining chemicals were purchased from Lachema, Brno.

Preparation of liver extracts. Male guinea pigs weighing 250—400 g were used in the experiments. The post-mortem proteolytic processes were studied under conditions identical to those described earlier[1]. Samples (1 g) of the liver were homogenized with cooling in a glass blendor with 10 ml of 0·1M phosphate buffer at pH 6·9. The homogenate was centrifuged (8000 *g*, 10 min, 0°C) and the supernatant was purified by ultrafiltration through a membrane filter (Synpor 6, mean pore size 0·4 μ). The liver extract yielded the same elution pattern as the original supernatant on gel filtration, yet the decrease of the flow rate during the separation was almost eliminated.

Gel chromatography on Sephadex G-100. The liver extracts were chromatographed in a K 15/90 column. A column bed of 1·5 × 80 cm and a flow rate of 30 ml/h were used. The samples were

eluted by 0·1M phosphate buffer at pH 6·9 in a total volume of 200 ml. For the determination of the approximate molecular weight of proteins contained in the liver extracts, the Sephadex G-100 column was calibrated with 6 protein standards in 4 chromatographic runs (Fig. 1). The standards were applied as 10 mg samples, the proteins of the liver extracts as 30 mg samples. The total volume of the sample was adjusted to 3 ml. The effluent was evaluated by a UV-analyzer at 254 nm, by absorbance measurement at 280 nm, and by the ninhydrin[2] and biuret reaction[3].

Gel chromatography on Sephadex G-25. The gel chromatography was allowed to proceed in the K 25/45 column (Pharmacia, Uppsala, 2·5 × 40 cm, flow rate 36 ml/h). The samples of the liver extracts were eluted with 350 ml of 0·1M phosphate buffer at pH 6·9. The column bed was calibrated by two peptides in orienting experiments and the elution volumes of an amino-acid mixture and of the individual aromatic amino acids were determined. The sample volumes were adjusted to 5 ml. The effluent was evaluated as described above for gel chromatography on Sephadex G-100.

Determination of free amino acids. The samples (1 g) of the liver tissue were homogenized in a glass blendor with 10 ml of 1% picric acid. The precipitated proteins were centrigufed off and picric acid removed on a column of Dowex 2 × 8 (100—200 mesh). The column bed (1 × 4 cm) was washed with 15 ml of 1M-HCl and then with distilled water to neutrality before the application of the sample. After the sample had passed through the column, the bed was washed at a low flow rate four times with 3 ml of 0·02M-HCl and amino acids were thus displaced. The eluate obtained was rotary evaporated to dryness and analyzed on an automatic amino acid analyzer (Type 6030, Instrument Development Workshops, Prague). A fresh bed of Dowex was used for each sample.

Determination of bound amino acids. The liver tissue (0·1 g) was hydrolyzed in 6M-HCl, at 110°C for 24 h. The hydrolysate was filtered and evaporated to dryness. The dry residue was dissolved in 30 ml of buffer at pH 2·2 used as sample buffer in the amino-acid analyzer. The samples were then analyzed as described for the determination of free amino acids. The dry weight was de-

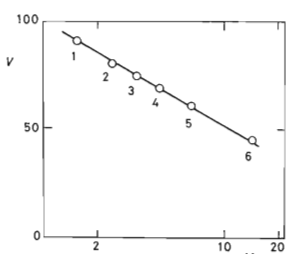

FIG. 1

Calibration of Sephadex G-100 Column for Determination of Molecular Weights of Proteins

Chromatographic Column K 15/90 Pharmacia, Uppsala (1·5 . 80 cm). Elution with 0·1M phosphate buffer at pH 6·9. Flow rate 30 ml/h. *V*, elution volume in ml; *M*, log of molecular weight × × 10^4 of protein on logarithmic scale. **1** α-Lactalbumin; **2** chymotrypsin; **3** β-lactoglobulin, **4** egg albumin, **5** serum albumin, and **6** alcohol dehydrogenase.

termined by drying of the material examined to constant weight at 105°C. The total nitrogen was determined by the Kjeldahl method after mineralization of the samples. The protein content of fractions from gel chromatography was determined spectrophotometrically[4].

RESULTS AND DISCUSSION

Chromatography of Liver Extracts on Sephadex G-100

The liver extracts prepared from fresh liver and from liver 24 h post-mortem were analyzed on Sephadex G-100. The results of these analyses expressed by absorbance curves at 254 nm are shown in Fig. 2. Since the liver extracts also contained nucleic acids in addition to proteins, these results were compared with the absorbance curves at 280 nm. The elution diagrams obtained at the two wavelengths are similar and therefore the protein content of the fractions can be evaluated by recording their absorbance at 254 nm. The content of nucleic acids and of their degradation products in the individual protein fractions is shown in Table I. The values are calculated from the absorbance values at 280 and 260 nm. The gel chromatography on Sephadex G-100 of extracts prepared from fresh and dying liver was repeated several times and always similar elution diagrams with identical positions of the maximums were obtained. Fraction 1 corresponds to a mixture of proteins of high molecular weights exceeding 150000. Fraction 2 contains proteins of molecular weights around 80000. The fraction designated 3 in the elution diagram contains predominantly amino acids and peptides. The elution pattern of gel chromatography of the liver extracts 24 h post-mortem showed the same peaks as the elution pattern of the extract of fresh liver, however one additional small peak designated 4 can be observed (Fig. 2). This peak can be seen also when the effluents are evaluated by the ninhydrin reaction. The fraction corresponding to peak 4 contains proteins of molecular weight around 18000. The fraction of high molecular weight proteins in the extract prepared from the liver 24 h post-mortem in always a little smaller whereas fraction 3 is larger.

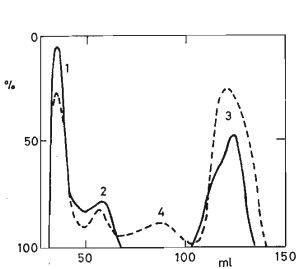

FIG. 2
Chromatography of Liver Extracts on Sephadex G-100

The experimental conditions are identical to those given in the legend to Fig. 1. ———, extract of fresh liver, – – – – – extract of liver 24 h post-mortem. 1, 2, 4, Protein fractions, 3 amino acids and peptides. ml, Elution volume; %, transmittance.

TABLE I

Gel Chromatography of Liver Extracts on Sephadex G-100
The values given correspond to the experiments shown in Fig. 2.

Designation of fractions	Elution volume ml	Main components of fractions	Approximate molecular weight	$\frac{A_{280}}{A_{260}}$	Nucleic acids %
		Fresh liver			
1	35	proteins	>150 000	0·94	4·0
2	57	proteins	80 000	1·08	2·5
3	120	amino acids and peptides		0·42	20·0
		Liver 24 h *post-mortem*			
1	35	proteins	>150 000	0·91	4·4
2	56	proteins	80 000	1·05	2·8
4	87	proteins	18 500	0·66	13·0
3	120	amino acids and peptides		0·74	8·6

Changes in Protein Concentration in Soluble and Insoluble Liver Fraction

The liver proteins are distributed by centrifugation of the liver homogenate under the conditions described between the liver supernatant and the insoluble sediment. As can be seen from Table II, the content of insoluble proteins in the sediment decreases; by contrast, proteins are supplied to the stock of soluble proteins of the extract, which is continuously decreased by the action of intracellular proteinases.

Chromatography of Liver Extracts on Sephadex G-25

The liver extracts which had been prepared from fresh liver and from liver 10 and 24 h post-mortem under the experimental conditions described were analyzed by gel chromatography on Sephadex G-25. The effluents were evaluated by continuous absorbance measurement at 254 nm (Fig. 3), at 280 nm, by the biuret reaction (Fig. 4) and by the ninhydrin reaction. Four fractions (Fig. 3) can be seen on the elution profile.

Fraction 1 contains proteins as evidenced by the elution volume and by the positive biuret and ninhydrin reaction. The elution volume of this fraction is in the case of the extracts of liver 10 and 24 h post-mortem slightly shifted toward a higher

value; this indicates a certain increase of the concentration of proteins of lower molecular weights. Fraction 2 involves peaks designated 2a and 2b. This fraction contains peptides as evidenced by its elution volume, a positive biuret (Fig. 4) and ninhydrin reaction. The elution volume of the fraction (150 ml) indicates that this

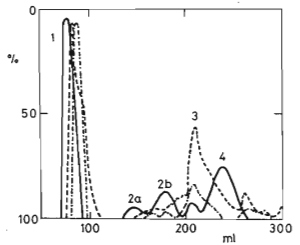

FIG. 3

Chromatography of Liver Extracts on Sephadex G-25

Chromatographic Column K 25/45 Pharmacia, Uppsala (2·5 × 40 cm). Elution with 0·1M phosphate buffer at pH 6·9. Flow rate 36 ml/h. ——— Extract of fresh liver, –·–·–·– extract of liver 10 h post-mortem, – – – – – extract of liver 24 post-mortem. 1, Protein fractions; 2a, 2b, peptide fractions; 3, fractions of amino acids and smallest peptides, and 4, fractions not containing amino acids; ml, elution volume; %, transmittance.

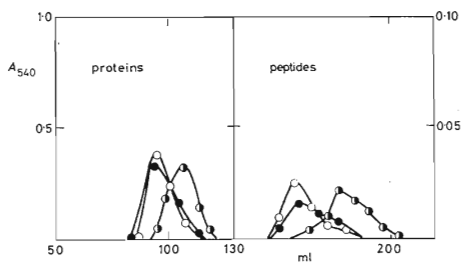

FIG. 4

Chromatography of Liver Extracts on Sephadex G-25 Evaluated by Biuret Reaction

The experimental conditions are identical to those given in the legend to Fig. 3. ●, extract of fresh liver, ○, extract of liver 10 h post-mortem, ◑, extract of liver 24 h post-mortem.

TABLE II

Changes in Concentration of Proteins in Soluble and Insoluble Liver Fraction

The insoluble liver fraction contains proteins of the sediment after the centrifugation of the liver homogenate. The soluble fraction contains proteins of the liver extract. These results are arithmetic means from 3 experiments

Period post mortem h	Protein content, %	
	insoluble fraction	soluble fraction
0	100	100
12	89	102
24	81	109

TABLE III

Concentration of Free Amino Acids in Fresh Liver and 24 h Post-mortem at 37°C in Relation to their Quantity Bound in Liver Tissue

The concentration of amino acids is given in μmol in 1 g of fresh tissue. [a] amino acids in the digest minus free amino acids in the fresh tissue; [b] free amino acids 24 h post-mortem minus free amino acids in the fresh tissue.

Amino acids	Digest	Fresh tissue	Content of liver proteins[a]	Content 24 h post-mortem	Quantity post-mortem[b]
Lys	20·6	0·65	19·95	1·16	0·51
His	9·7	0·16	9·54	0·42	0·26
Asp	28·6	0·71	27·89	2·50	1·79
Thr	16·9	0·22	16·68	1·65	1·43
Ser	19·2	0·35	18·85	1·93	1·58
Glu	40·5	0·93	39·57	3·18	2·25
Pro	13·1	0·42	12·68	1·25	0·83
Gly	29·7	0·90	28·80	3·08	2·18
Ala	32·0	0·78	31·22	3·50	2·72
Val	12·2	0·58	11·62	1·35	0·77
Met	1·5	0·17	1·33	0·21	0·04
Ileu	10·3	0·52	9·78	1·13	0·61
Leu	22·7	0·85	21·85	2·40	1·55
Tyr	8·2	—	8·20	0·60	0·60
Phe	10·8	0·34	10·46	0·90	0·56

fraction contains peptides of mean molecular weight of approximately 1500. The second subfraction, designated 2b, contains peptides of even lower molecular weights. Hence, the fresh liver tissue contains peptides of molecular weights lower than 1500 and at very low concentrations. The level of the peptides slightly increases (Fig. 4) during the middle phase of post-mortem proteolysis (10 h) and the mean molecular weight is shifted to the relatively highest values. Peptides composed of a smaller number of amino-acids prevail during the subsequent stage of the post-mortem process. Fraction 3 contains peptides composed of a few amino acids. These compounds are cumulated in the liver tissue during the post-mortem proteolysis. The relatively highest content of oligopeptides shows the extract of the liver 10 h post-mortem. Fraction 4 gives a negative biuret and ninhydrin reaction.

The results given above permit us to assume that the intracellular proteinases degrade the liver proteins to amino acids and oligopeptides without any significant cumulation of the intermediary products in the tissue. This finding can be explained in two manners: either by the low specificity of intracellular proteinases unable to distinguish between the types of peptide bonds or by a combined action of the individual proteinases in the course of proteolysis. We prefer the second possibility which is in accordance with the findings of Iodice and coworkers[5] that a mixture of cathepsin A and D shows an activity twice higher than the sum of the activities of both enzymes acting separately. This increase of proteolytic activity can be explained by assuming that one enzyme prepares the substrate for the other one. A similar mutual interaction of cathepsins A and C and of cathepsins B and D has also been postulated by Ali and Evans[6].

Changes in Concentrations of Free Amino Acids in Liver Tissue during post-mortem Proteolysis

The content of free amino acids occurring in the fresh liver tissue and in the liver tissue after a 24 h post-mortem period was determined. In addition, the amino-acid composition of liver proteins was also determined (Table III). The total quantity of the free amino acids in the fresh liver tissue was approximately 2·8% of the total quantity of amino-acids bound in the liver proteins. Their quantity increased to 9·4% after the 24 h post-mortem period. This finding is in accordance with the results of our preceding study in which the changes in the concentration of amino acids during post-mortem proteolysis were examined after another isolation procedure[7]. If we express the total quantity of amino acids released during the 24h post-mortem period in per cent of their quantity bound in the liver proteins we arrive at 6·6%. As can be seen from Table III, most amino acids are released in proportion to their content in the liver proteins.

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