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Changes in some enzyme activities and DNA content in frozen stored and freeze-dried bovine thymus

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Abstract

The effect of the preservation method and storage time of bovine thymus on the activity of adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP), as well as on the quantity changes of desoxyribonucleic acid (DNA), was studied in this work. Fresh bovine thymus was divided into 3 groups. The first one was frozen and stored at −12°C, the second at −30°C, and the third was freeze-dried. The last was placed in air-tight containers and stored at 4°C. Every 2 months during 20 months of storage, the activity of ADA and PNP by the Kalckar method and DNA content by the diphenylamine method were determined. Lowest activities of ADA and PNP were in freeze-dried samples while the highest were found for the frozen samples stored at −12°C. DNA content was at nearly the same level throughout the storage time. If fresh thymus is not available for the production of drugs containing the analysed enzymes and DNA, it is sufficient to freeze it. In this state the gland remains stable for nearly 2 years.

1. Introduction

Bovine thymus is one of the inedible by-products in the meat industry. The presence of biologically active proteins and enzymes makes it a desirable raw material for pharmacists. Therefore, on the basis of its extracts the production of biopreparates has been developed. Showing hormonal activity, these are applied in the therapy of immunological deficiency (Dąbrowski, 1987; Pesic, 1989/90; Pesic et al., 1991).

The gland contains a significant amount of desoxyribonucleic acid (DNA) which is 20–40 times higher than that found in muscle tissue (Synowiecki & Shahidi, 1992; Tajima, Matsumoto-Koyama, & Nishio-Kozu, 1989). Hence, in medicine, thymus extracts containing DNA are used for the treatment of children suffering from Down's syndrome or cerebral palsy (Czaplicki, Błońska, Pesic, Kalamoniak, & Trzczcinska-Fajfrowska, 1993; Pesic, 1989/90).

Other compounds of the bovine thymus are also of interest to researchers. These are: adenosine/2'-deoxyadenosine aminohydrolase, EC.3.5.4.4.(ADA), and nucleoside phosphorylase, EC. 2.4.2.1. (PNP). ADA belongs to the class of nucleoside deaminases which catalyse oxygen deamination of adenosine and 2'deoxyadenosine to inosine. Nucleoside phosphorylase shows phosphorylitic activity and catalyses the reversible nucleoside phosphorylation to the free purine bases. The enzymes have been found in almost all the tissues of mammals (Martinez, Zumalacarregui, Diez, & Burgos, 1984; Spivey & Snow, 1989). However, in practice, only the thymus may be considered as a source of their recovery. The mentioned enzymes have been the subject of many studies concerning mainly erythrocytes. As a result, a dependence has been found between the hereditary deficiency of these enzymes in the human body and the occurrence of certain immunological diseases (Giblett, 1979, 1991).

TFX-Thymomodulin, Thymex L and other mature thymus extracts have found clinical application in building up resistance in children with inborn immmune deficiency (Aiuti et al., 1979; Czaplicki et al., 1993; Goldstein et al., 1976; Wara & Ammann, 1978).

Fresh bovine thymus, as a raw material for the pharmaceutical industry, is not always available. Usually, it is frozen and stored in this state before processing. It would be interesting to learn what are the changes taking place during such storage, as well as how long such a raw material is stable and may be used for the production of biopreparations. Moreover, the usefulness of freeze-drying as a method of bovine thymus preservation has not yet been investigated.

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Therefore, in this investigation, the changes in DNA content and ADA and PNP activity were studied during freezing and frozen storage of bovine thymus at different temperatures. The results were also compared with those obtained for freezed-dried samples to evaluate the usefulness of freeze-drying as a method of preservation.

2. Materials and methods

2.1. Materials

Fresh bovine thymus (about 4 kg), purchased from the slaughterhouse in Kraków, was the raw material for analysis. After its delivery to the laboratory, the material was divided into 3 parts.

Part I (about 1 kg.), after grinding ($\phi = 1$ cm), was frozen at -30°C for 8 h and then freeze-dried in a type OE 950 LABOR MIM freeze-drier. The parameters of the process were as follows: temperature of the plate 30°C; pressure in the chamber under 0.5 mm Hg. Both temperature and pressure were then monitored throughout the 48 h of the freeze-drying process. Freeze-dried samples were stored at 4°C in hermetic containers in the refrigerator. Before analysis, the samples were rehydrated for 8 h at 4°C by the addition of deionized water in an amount equal to that released during freeze-drying.

The remainder of the glands was cut into smaller pieces and mixed to constitute a raw material. Next, portions of about 120 g were prepared, packed in polythene bags, then frozen at -12 and -30°C (11 portions at each temperature) in a freezer without air blasting. Under these conditions the material was stored for 20 months. Investigations were carried out on the fresh bovine thymus, immediately after freezing and freeze-drying, and then every 2 months. Before the analyses the samples were thawed in a microwave oven until a temperature of 0°C in their thermal centre was reached.

2.2. Methods

The content of desoxyribonucleic acid, given in mg of DNA/g of tissue, was determined by the diphenylamine method (Schneider, 1957).

Extracts for determining the activity of enzymes and protein content were prepared as follows. Ground bovine thymus ($\phi = 1.5$ mm) was mixed with a cool solution of 0.2 M KCl in the proportion 1: 10 for 15 min. The mixture was then filtered through cloth. Finally, the protein content was determined by the biuret method (Snow, 1950). The same extracts were used to determine the activity of ADA and PNP by the Kalckar method (Kalckar, 1947, 1945) adapted by Thymoorgan GmBH Pharmazie and Co K.G. Vienenburg for the analysis in thymus extracts. To measure ADA activity, the following assay mixture was prepared: 2.88 cm^3 0.1 M phosphate buffer, pH 7.4, 0.1 cm^3 of adenosine solution with a concentration of 1.4 mM and 0.02 cm^3 of enzymatic extract. The whole was then stirred and the decrease in absorbance within 5 minutes was read at a wavelength of 265 nm using a VSU 2-P Carl Zeiss Jena spectrophotometer. As control, a solution containing 2.88 cm^3 of buffer, 0.1 cm^3 of adenosine and 0.02 cm^3 of deionized water was prepared. Analyses were carried out at a temperature of 25°C using a 1 cm path length quartz cuvette. The ADA activity was expressed as specific activity regarding protein content and was calculated as mU/mg of protein (nmol/min/mg of protein).

For measurement of PNP activity, an assay mixture was prepared containing 3.0 cm^3 0.1 M of phosphate buffer pH 7.4, 0.1 cm^3 of inosine solution of concentration 7.3 mM, 0.01 cm^3 of xanthinoxidase suspension (0.4–1.2 U/mg), and 0.05 cm^3 of enzymatic extract. The mixture was then stirred and increased absorbance was monitored during 5 min at a wavelength of 293 nm at 25°C. The control sample contained 3.0 cm^3 of buffer, 0.1 cm^3 of inosine, 0.01 cm^3 XOD suspension, and 0.05 cm^3 of deionized water. The PNP activity was calculated and expressed similarly as ADA activity.

Data concerning ADA and PNP activities as well as DNA quantities were subjected to two-way analysis of variance using the computer program Excel 5.0.

3. Results and discussion

3.1. Changes in ADA activity

The changes in ADA activity in frozen and freeze-dried bovine thymus are given in Table 1. It was observed that both the freezing process and freeze-drying resulted in decreased enzyme activity. The greatest decrease was found in the case of freeze-drying, while after freezing at -12°C it was statistically non-significant. A similar effect was reported by Vitolo, Breda, Ronaldo, and Duranti (1991) who investigated changes in invertase activity caused by freezing and freeze-drying. Depending on the manner of freezing, no decrease or only a slight one was observed in its activity while freeze-drying reduced the enzyme activity significantly. During the sublimation process the ionic strength of the solution increases, and there are changes in the configuration of enzyme proteins and pH, which may lead to a decrease in enzyme activity. It has been found by numerous researchers that freeze-drying may bring about various changes, depending on the kind of enzyme. For instance, ATP-ase is resistant to this process (Tsvetkow & Naidenova, 1987) while glucose oxidase requires the addition of stabiliser (Halpern, 1981).

Phosphofructokinase too is totally inactivated after freeze-drying and dissolution (Carpenter, Crowe, & Crowe, 1987). A fall in ADA activity in freeze-dried samples was also observed during the whole storage period. In the 20th month of storage, freeze-dried samples had 73% of the activity determined at the beginning (after preservation). Different results were found for frozen bovine thymus. Throughout the storage period, ADA activity in thymus stored at −12 and −30°C was, respectively, approximately 36 and 23% higher than in freeze-dried samples. Moreover, it was also noted that, at −12°C, ADA activity even exceeded the level determined in fresh gland in a few months. However, when compared with the results obtained for fresh bovine thymus, the mean ADA activity was about 16% lower at the temperature −30°C, while at −12°C the activity was almost identical. The two-way analysis of variance showed statistically significant differences among the three preservation methods ($\alpha = 0.05$ and 0.01) and holding time (only for $\alpha = 0.05$).

Freeze-drying, as a preservation method, is especially recommended for all biologically active protein compounds which maintain a stable, unchanging level of activity. It should be pointed out, however, that these are substances of very high purity, not a tissue in which many compounds may interact giving untypical changes. The fact that ADA maintained a higher activity in frozen samples could be a consequence of the location of the enzymes.

Enzymes located in cellular structures may retain their activity during freezing and even show a significant increase as a result of their release from these structures (Hamm, 1979). For example, ADA is located partly in the cytoplasm and partly in various subcellular structures such as mitochondria, cytosols, or cell nucleus. This may explain why ADA maintains a higher activity during storage in a frozen state. (Dinjens et al., 1989).

The activity of this enzyme was higher in bovine thymus stored at −12°C than in that stored at −30°C, which was probably caused by the fact that, at a higher temperature, enzyme activators may be released because of more extensive tissue damage.

Investigations carried out by Fabianowska-Majewska and Greger (1992) indicated a significant stability of ADA. It has been demonstrated that erythrocyte samples stored at −85°C retain stable enzyme activity even for 2 years. The enzyme was also resistant to high temperatures.

3.2. Changes in PNP activity

The effect of preservation methods and time of storage on the PNP activity is seen in Table 2. Freezing at −12 and −30°C as well as freeze-drying led to a decrease in activity, similar to that observed for ADA. However, the nearly 37% decrease in activity (in comparison with the activity determined in fresh gland—treated as 100%) was equally rapid for all the applied versions of preservation. During 20 months of sample storage in a frozen state, at both temperatures, their PNP activity was higher than in freeze-dried samples. For the temperatures −12 and −30°C, the mean difference was about 30 and 15%, respectively. It must be pointed out that, at −12°C, a few times during the storage period, PNP activity almost approached the activity determined in fresh gland. Two-way analysis of variance confirmed the significant effect of preservation methods and storage time on PNP activity ($\alpha = 0.05$ and 0.01). Hence, the behaviour of PNP in both processes as well as the reasons for such a behaviour are similar to those for ADA.

In spite of the fact that the enzymes belong to two different classes, they are similarly located in a cell and cooperate with each other (Peters & Veerkamp, 1983). In this context the reasons for the observed changes could be similar.

Table 1
Specific activity of ADA in bovine thymus expressed in mU/mg protein (mean of three samples ± SD)

Time of storage	Preservation method		
	−12°C	−30°C	FD[a]
Fresh		392 ± 40.1	
after preservation	389 ± 39.0	309 ± 35.7	254 ± 41.8
2 months	367 ± 66.3	361 ± 41.5	260 ± 33.1
4 months	393 ± 45.5	333 ± 32.0	240 ± 31.1
6 months	386 ± 48.6	361 ± 40.5	274 ± 34.3
8 months	452 ± 52.0	324 ± 48.1	298 ± 30.2
10 months	427 ± 38.0	342 ± 43.9	245 ± 31.5
12 months	466 ± 44.1	353 ± 48.3	271 ± 29.0
14 months	394 ± 55.2	261 ± 32.2	293 ± 26.1
16 months	338 ± 44.1	316 ± 36.7	243 ± 22.4
18 months	354 ± 34.5	272 ± 32.5	227 ± 31.0
20 months	390 ± 48.8	388 ± 42.9	187 ± 30.8

[a] Freeze-drying.

3.3. Changes in DNA content

The changes in DNA content in frozen and freeze-dried bovine thymus were within the range 27.8 to 34.9 mg DNA/g of tissue (Table 3). Freezing of the gland at both temperatures caused a decrease in its amount. The slight decrease in its content resulting from freeze-drying, was statistically non-significant. During storage, the DNA content fluctuated but, except for the thymus stored at −30°C and freeze-dried, the differences between the preservation methods were statistically non-significant. Namely, in freeze-dried thymus the DNA content was approximately 5% lower than that in the gland stored at −30°C. The difference was not great but statistically significant (for $\alpha = 0.05$ and 0.01). Finally, at the end of storage (20th month), no significant differences between DNA content in frozen and freeze-dried bovine thymus or in fresh gland were observed. It is evident from these results that long-term storage of the gland did not degrade DNA.

There is still a lack of information concerning DNA changes in frozen food. The effect of low temperatures was studied mainly in the context of mechanical damage to tissue by determining the content of DNA in the drip or from the aspect of mutagenic changes. Ashwood-Smith and Grant (1977), in his investigations on mutagenic interaction at low temperature, demonstrated a high resistance of DNA to freezing and thawing both *in vitro* and *in vivo*. These findings suggested that storage in a frozen state would not cause its degradation. The small percentage loss of DNA content in freeze-dried samples could probably be explained by better-maintained desoxyrybonuclease activity.

Table 2
Specific activity of PNP in bovine thymus expressed in mU/mg protein (mean of three samples ± SD)

Time of storage	Preservation method		
	−12°C	−30°C	FD[a]
Fresh		19.5 ± 1.81	
after preservation	12.4 ± 1.56	12.4 ± 1.34	12.1 ± 1.23
2 months	18.4 ± 1.80	16.8 ± 1.46	12.4 ± 1.13
4 months	17.7 ± 1.58	14.3 ± 1.21	13.4 ± 1.31
6 months	20.5 ± 2.49	16.9 ± 1.55	12.5 ± 1.20
8 months	21.8 ± 2.25	17.6 ± 1.55	11.5 ± 1.12
10 months	20.3 ± 2.12	16.0 ± 1.65	12.8 ± 1.17
12 months	22.5 ± 1.95	16.7 ± 1.66	13.7 ± 1.23
14 months	17.5 ± 1.85	14.3 ± 1.30	13.4 ± 1.36
16 months	15.3 ± 1.42	12.8 ± 1.27	13.7 ± 1.17
18 months	18.6 ± 1.85	14.4 ± 1.45	14.2 ± 1.35
20 months	20.8 ± 2.21	19.5 ± 1.73	15.0 ± 1.55

[a] Freeze-drying.

Table 3
The content of DNA in bovine thymus expressed in mg DNA/ g tissue (mean of three samples ± SD)

Time of storage	Preservation method		
	−12°C	−30°C	FD[a]
Fresh		33.9 ± 1.51	
after preservation	31.0 ± 1.39	29.7 ± 1.00	33.1 ± 1.69
2 months	30.1 ± 1.38	30.7 ± 1.36	31.4 ± 1.57
4 months	30.3 ± 1.25	34.5 ± 1.46	33.4 ± 1.67
6 months	33.7 ± 1.73	34.5 ± 1.59	32.6 ± 1.60
8 months	30.6 ± 1.80	30.8 ± 1.20	28.4 ± 1.18
10 months	33.0 ± 1.37	32.2 ± 1.60	29.2 ± 2.05
12 months	30.0 ± 1.73	32.3 ± 1.83	33.7 ± 1.61
14 months	32.1 ± 1.44	30.6 ± 1.44	27.8 ± 1.93
16 months	32.6 ± 1.50	33.1 ± 1.34	28.4 ± 1.53
18 months	31.9 ± 0.96	34.9 ± 1.78	30.0 ± 1.43
20 months	32.4 ± 1.50	34.0 ± 1.70	32.4 ± 1.50

[a] Freeze-drying.

4. Conclusions

The obtained results indicate that the method of preservation has little effect on DNA content, while both freeze-drying and freezing at −30°C lead to a decrease in ADA and PNP activity. In general, freezing which is the commonly used method of bovine thymus preservation in drug production, does not lead to any significant reduction of the analysed factors, in spite of such a long holding time. Moreover, it has been found that it is an even better method than freeze-drying. Bovine thymus stored in a frozen state for almost 2 years is still a good, valuable raw material for the pharmaceutical industry. These results confirm the earlier observations concerning the stability of ADA and PNP. They may be used to characterize bovine thymus as a raw material for the pharmaceutical industry.

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