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Preservation of the proteolytic activity of a bovine spleen lysosomal-enriched extract using various freezing conditions

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Abstract

The preservation of the proteolytic activity of a bovine spleen lysosomal-enriched (BSLE) extract was investigated. The BSLE extract (pH = 5.8), was subjected to storage under different conditions: refrigeration at 0°C for 60 days; freezing at −20°C —either directly or previously frozen in liquid nitrogen—, −80°C and in liquid nitrogen; freeze-drying and stored at 0°C; and freezing at −20°C or in liquid nitrogen in the presence of glycerol and sorbitol as cryoprotectants. Freezing at low temperatures (-80°C and in liquid nitrogen) was most effective for preserving about 100% of the initial activity of all cathepsins (B, B+L and D), as well as the activity of the extract on myofibrils, for two years. Freezing at −20°C, on the contrary, led to significant ($P < 0.01$) losses of activity. Freeze-drying was able to preserve cathepsin activity, while it failed to maintain activity on myofibrils. Both cryoprotectants sorbitol and glycerol significantly ($P < 0.01$) enhanced enzyme preservation, particularly cathepsin D and the activity on myofibrils, even at a freezing temperature of −20°C.

Keywords: Spleen; Proteases; Cathepsins; Myofibrillar proteins; Freezing; Cryoprotectants

1. Introduction

Lysosomes are present in almost all mammalian cells and tissues, but they are particularly abundant in spleen and liver. A great variety and quantity of enzymes is found in the lysosomes. Most of them have a pH optimum in the acid range, although some enzymes have activity at neutral pH. The enzyme mixture includes proteases (cathepsins), lipases, glycosidases, nucleases, phospholipases, phosphatases and sulphatases (Dean and Barrett, 1976) [1]. Cathepsins constitute the most important proteolytic intracellular system (Asghar and Bhatti, 1987) [2].

Bovine spleen is known to be a very good source of many cathepsins, mainly B, L, D, H, N and S. Melendo et al. (1998a) [3] reported the enzymatic characterization of a bovine spleen lysosomal-enriched (BSLE) extract. The extract showed a high activity of all investigated cathepsins (B, B+L, D, G and H), a considerable ability to degrade both myofibrillar proteins and collagen, as well as of exopeptidase; proteolytic activity was accompanied by lipase and esterase activities, and a minor peroxidase activity. It was therefore concluded that the bovine spleen lysosomal-enriched extract could be a useful tool for the tenderization and ripening of muscle foods such as meat, fish and their products. Artigas et al. (1996) [4] have also shown that bovine cardiac muscle is a good source of cathepsins for their use in food industrial technology.

Cohen et al. (1979) [5] showed that precooked freeze-dried beef treated with a spleen extract was more tender and uniform in texture. The same group proved also that the texture of muscle treated with lysosomal extract was similar to that of naturally aged meat (Robbins et al., 1979) [6]. We have reported the application of a lysosomal bovine spleen extract both to the tenderization of squid (Melendo et al., 1998b) [7] and of fresh beef steaks (Melendo et al., 1998c) [8]. Those results proved the suitability and usefulness of its use. The BSLE extract appears then to be of sufficient technological interest as to undertaking further research on its properties and applications.

The main problem associated to the industrial application of enzyme-enriched extracts is the loss of enzyme activity throughout storage. Indeed, proteases are known to loose

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their activity at a fairly fast rate. Therefore, a number of methods have been developed for the preservation of enzyme activity; these include freezing under varying conditions and freeze-drying, as well as the presence of different salts, surfactants or the use of cryoprotectants. However, the preservation of enzyme activities in lysosomal-enriched extracts has not been thus far investigated in depth. Alone a study on the preservation of a liver extract by using triton X-100 has been reported by Béchet et al. (1986) [9].

The aim of this research was to find out a reliable method for the preservation of the protease activity of a crude lysosomal-enriched extract obtained from bovine spleen. The following experimental conditions have been investigated: refrigeration at 0°C, freezing at −20°C, −80°C and in liquid nitrogen, freeze-drying and the use of glycerol and sorbitol as cryoprotectants.

2. Materials and methods

2.1. Preparation of the lysosomal-enriched extract

Bovine spleens were obtained immediately after slaughter; the lysosomal-enriched fraction was prepared as described by Lardeux et al. (1983) [10]. Briefly, spleen was minced and homogenized with a Potter homogenizer in 10 volumes of 10 mM potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA. The spleen homogenate was centrifuged 10 min at 1000 g and then 10 min at 4000 g. The supernatant was centrifuged at 20000 g for 20 min and the pellet was homogenized in 20 mM phosphate buffer (pH 5.8) and frozen at −20°C. Frozen homogenate was thawed after 48 h and the supernatant of the lysosomal-enriched extract recovered after 20 min centrifugation at 100000 g.

2.2. Treatment of samples for preservation

One ml aliquots of the bovine spleen lysosomal-enriched extract (BSLE extract) were prepared. They were subjected to the following treatments: a) storage in an ice-water bath in a cold room at 0°C for 60 days; b) storage in a freezer at −20°C for 2 years; c) storage in a freezer at −80°C for 2 years; d) storage in a container filled with liquid nitrogen for 2 years; e) freezing with liquid nitrogen and storage in a freezer at −20°C for 2 years; and f) freeze-drying and storage in a cold room at 0°C for 2 years. For the experiments regarding the use of cryoprotectants, either glycerol or sorbitol were added to 1 ml aliquots of the BSLE extracts, to reach a final concentration of 4%. They were subjected to the following treatments: a) storage in a freezer at −20°C for 2 years; b) storage in a container filled with liquid nitrogen for 2 years

Aliquots were taken as needed (see the Results section) from their storage conditions along the whole maintenance period. Each aliquot, in duplicate, was used for an individual enzyme activity assay.

2.3. Assay of enzyme activities

Activity of cathepsins B, H and L was assayed according to Barrett and Kirschke (1981) [11]. Cathepsin B was assayed specifically with *N*-CBZ-L-arginyl-L-arginine 7-(4-methyl) coumarylamide (Z-Arg-Arg-NHMEC) (Sigma), Cathepsins B + L were assayed with the common substrate *N*-CBZ-L-phenylalanyl-L-arginine 7-(4-methyl) coumarylamide (Z-Phe-Arg-NHMEC) (Sigma) and Cathepsin H was assayed specifically with L-arginine 7-(4-methyl) coumarylamide (Arg-NHMEC) (Sigma). BSLE (10 μl) and substrates (at a final concentration of 5 μM) were incubated 30 min at 37°C and pH 5.5 (cathepsin H at pH 6.8). The intensity of fluorescence of the mixture was measured at an excitation wavelength of 350 nm and an emission wavelength of 460 nm. One unit of activity was defined as the amount of enzyme hydrolyzing 1 μmol of substrate per min and g of spleen at 37°C. The assay for cathepsin B activity was carried out using 1 unit of purified cathepsin B (Sigma), in addition to the BSLE extract, at each day of analysis throughout the 2 years of storage, in order to be used as standard reference.

Cathepsin D was assayed using denatured bovine haemoglobin (Sigma). One ml of the incubation mixture contained 0.25 mmole formiate, 0.1% Brij, 0.05% haemoglobin and 10 μl of the extract, pH 3.5. After 1 h incubation at 45°C, the reaction was stopped by addition of 10% trichloroacetic acid (TCA), and the TCA-soluble peptides measured by the method of Lowry et al. (1951) using L-tyrosine as standard. One unit of activity was defined as the amount of enzyme releasing 1 μg of tyrosine per min and g of spleen at 45°C (Etherington et al., 1987). The assay for cathepsin D activity was carried out using 2,5 μg of purified cathepsin D (Sigma), in addition to the BSLE extract, at each day of analysis throughout the 2 years of storage, in order to be used as standard reference.

Exopeptidase activity was assayed in 1 ml 100 mM sodium acetate buffer, with 5 mM DTT and 1 mM EDTA, at pH 5, containing 10 mg/ml of myofibrillar proteins, incubated with 10 μl of spleen extract, at 30°C during 20 min. Using an aliquot of 0.2 ml, the reaction was stopped with 1.5 ml of ninhydrin (Doi et al., 1981) [17]. The mixture was incubated at 84°C during 5 min and cooled down quickly; absorbance was measured at 505 nm. One unit of activity was defined as the amount of enzyme releasing 1 μg of glycine per min and g of spleen at 30°C.

2.4. Activity on myofibrillar proteins

The extract was incubated with unspecific protein substrates in 100 mM sodium acetate buffer, containing 5 mM dithiothreitol (DTT) and 1 mM ethylenediaminetetraacetic acid (EDTA), at pH 6 (myosin was assayed at pH 5.5), at

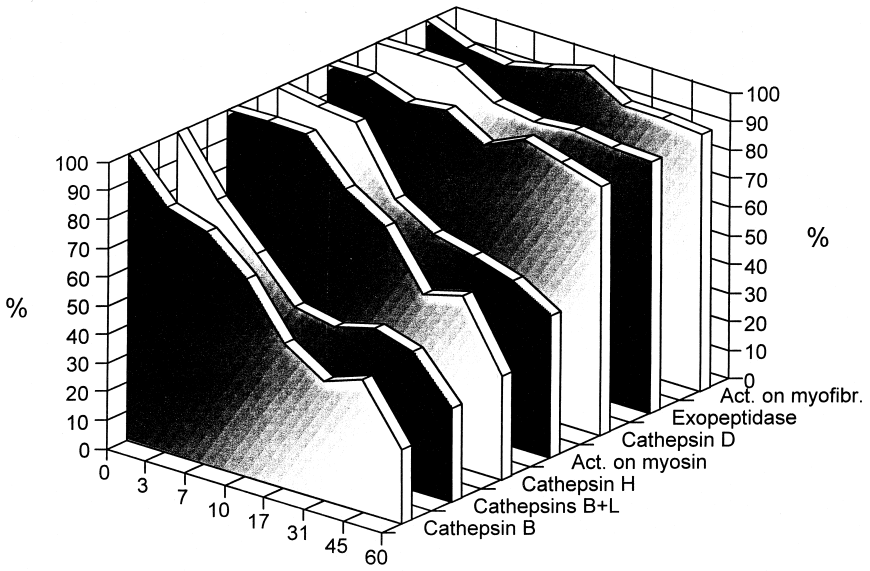

Fig. 1. Activity of cathepsins B, B+L, D and H, exopeptidase and effective proteolytic activity on myofibrils and purified myosin, all expressed as a percentage of initial value, throughout the storage of a bovine spleen lysosomal-enriched extract under refrigeration at 0°C.

30°C during 20 min. Substrates were either 10 mg/ml of myofibrillar proteins, obtained by the method described by Olson et al. (1976) [18] or 3 mg/ml of myosin, purified according to the method described by Quass and Briskey (1968) [19]. The reaction was stopped by addition of 5% TCA; the mixture was centrifuged 20 min at 1000 g and supernatant absorbance was measured at 278 nm. One unit of activity was defined as the increase of 0.001 units of absorbance at 278 nm per min and g of spleen at 30°C.

2.5. Statistical analysis

The significance of differences among samples at each day of storage was determined by analysis of variance using the Least Square Difference method of the General Linear Model procedure of SPSS (SPSS, 1995) [20]. Differences were considered significant at the $P < 0.01$ level

3. Results

3.1. Storage of the BSLE extract at 0°C

Samples of the BSLE extract were stored under refrigeration at 0°C in an ice bath for 60 days. Fig. 1 shows the activity profiles, expressed as a percentage of the initial value, of the following enzymes or proteolytic activities: cathepsins B, B+L, D and H, exopeptidase and effective activities of the extract on myosin and myofibrillar proteins, during the storage period. For absolute activity values, see Melendo et al. (1998a) [3].

All three catheptic activities B, B+L and H showed a fairly rapid decrease, with final values after 60 days of storage within the range 20–30% of initial activity of the extract. On the contrary, cathepsin D exhibited a considerable ability to maintain its original activity in the BSLE extract, showing final values above 80%. The extract also retained a high exopeptidase activity after 60 days of storage, above 80% of its initial value.

The effective activity of the extract on muscle proteins presented remarkable differences. In fact, proteolytic activity on myofibrils remained almost constant along the 60 days of storage, always above 90% of initial values, while activity on purified myosin decreased steadily to a final value of about 40%.

3.2. Storage of the BSLE extract under freezing conditions

In a second experiment, samples of the BSLE extract (pH = 5.8) were stored for 2 years under the following conditions: −20°C, −80°C, in liquid nitrogen, frozen in liquid nitrogen and stored thereafter at −20°C, and freeze-dried and stored at 0°C. Activity of cathepsins B, B+L and D, and the effective proteolytic activity on myofibrils were recorded along the storage period. The results, shown in Fig. 2, are in all cases expressed as a percentage of the initial value

Fig. 2 (A) shows the results obtained for cathepsin B. Storing the extract at −20°C resulted in an activity decrease to a final value of 60% of the initial value, which was in fact already reached after one year of storage. The same rate of reduction was evident in samples frozen in liquid nitrogen and stored thereafter at −20°C. However, the extract samples which were subjected to freeze-drying and stored at 0°C maintained an activity of about 100% for the two-year period. Surprisingly, samples stored at −80°C and in liquid nitrogen showed an increase in cathepsin B activity, which

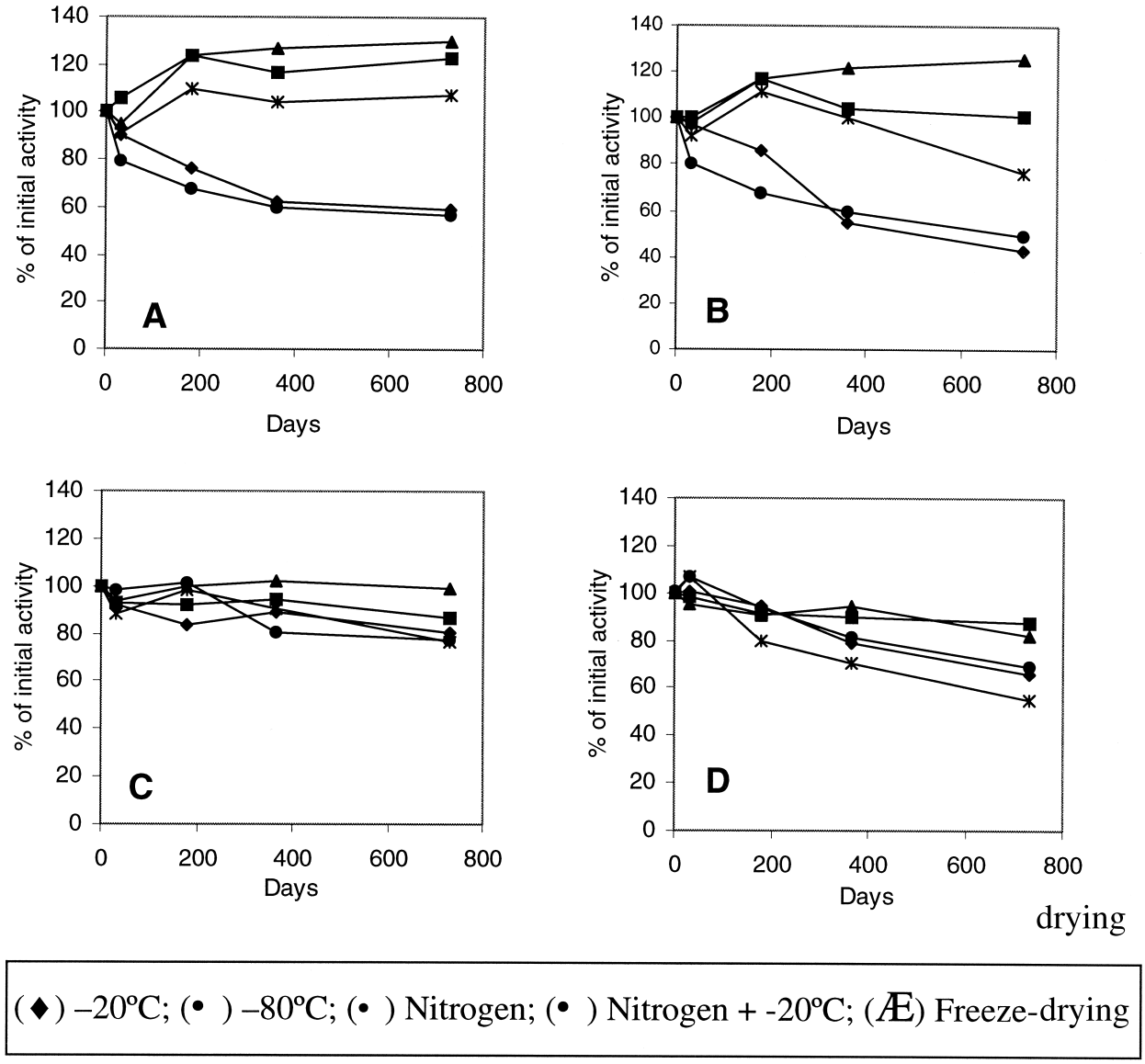

Fig. 2. Activity of cathepsin B (A), cathepsin B+L (B), cathepsin D (C) and effective proteolytic activity on myofibrils (D), all expressed as a percentage of initial value, throughout the storage of a bovine spleen lysosomal-enriched extract under different freezing conditions.

reached about 120–130% of their initial values from the sixth month of storage onwards. Differences were statistically significant ($P < 0.01$).

Cathepsin B+L activity (Fig. 2-B) presented a similar behavior to that of cathepsin B alone, but with final values slightly lower. The steady decrease of the samples stored at −20°C, either directly unfrozen or previously frozen in liquid nitrogen, reached final values of 40–50%. The extract samples freeze-dried and stored at 0°C maintained an activity around 100% for the first year, while it decreased to about 80% at the end of the storage period. Samples stored at −80°C maintained an activity slightly above 100%, while those stored in liquid nitrogen increased their activity up to 130%, in the same manner as cathepsin B alone. All the differences described above were statistically significant ($P < 0.01$).

Activity of cathepsin D during frozen storage under the different conditions (Fig. 2-C) showed a completely contrasting behavior. First, there were no significant differences among freezing treatments, with the exception of samples stored in liquid nitrogen, whose activity was significantly higher ($P < 0.01$) than those of samples stored at −20°C. Second, all of them demonstrated a small but steady loss of activity, reaching final values after two years of storage of 80–90% of its initial activity, with the exception again of the extract stored in liquid nitrogen, which remained around 100%.

Regarding the effective proteolytic activity of the BSLE extract on myofibrils, results displayed in Fig. 2 (D) demonstrated an intermediate behavior between those of cathepsin B and B+L and cathepsin D. As a matter of fact, all of the freezing and storage treatments resulted in a steady

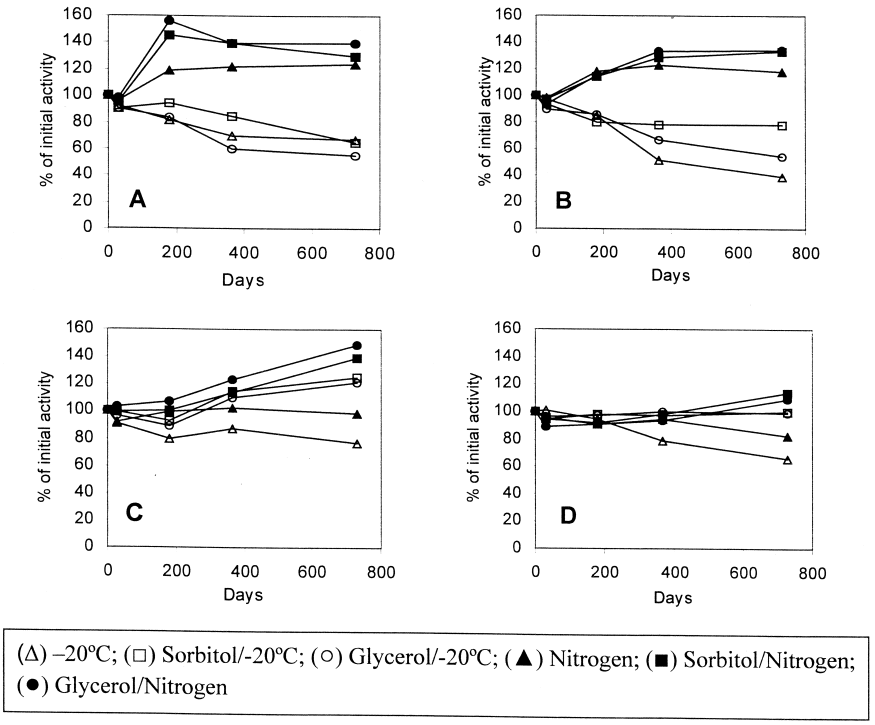

Fig. 3. Activity of cathepsin B (A), cathepsin B+L (B), cathepsin D (C) and effective proteolytic activity on myofibrils (D), all expressed as a percentage of initial value, throughout the storage of a bovine spleen lysosomal-enriched extract under different freezing conditions in the presence of cryoprotectants.

decrease of initial activity. However, significant differences ($P < 0.01$) among treatments were evident. All samples subjected to storage at −20°C, either directly or frozen in liquid nitrogen, and to freeze-drying, decreased to reach final values of 60–70% of initial activity. On the other side, both samples stored at very low temperature, either −80°C or in liquid nitrogen, had a minor lessening of initial activity, which remained above 85% at the end of the two-year storage period.

3.3. Storage of the BSLE extract under freezing conditions with cryoprotectants

A third experiment was designed in order to assess the protective effect of cryoprotectants on the proteolytic activity of the BSLE extract stored under different freezing conditions. Storage conditions used were −20°C and in liquid nitrogen, and the cryoprotectants, selected among the most widely employed, were sorbitol and glycerol. Fig. 3 depicts all those results

Fig. 3 (A) shows the results concerning cathepsin B. As already stated in the preceding section, a highly significant difference in the extract activity was found between storage at −20°C and in liquid nitrogen, reaching final values of 70% and 130% of initial activity, respectively. The presence of cryoprotectants, either sorbitol or glycerol, did not exert any effect on the activity of the extract stored at −20°C. On the contrary, both of them led to a significant increase ($P < 0.01$) of the extract activity stored in liquid nitrogen throughout the storage period, reaching 140–150% of initial activity within the early phase (6–12 months); however, glycerol failed to significantly increase ($P > 0.01$) its activity at the term (2 years) of the storage.

Cathepsin B+L activity (Fig. 3-B) showed a fairly different behavior, since the presence of both cryoprotectants led to a significant increase ($P < 0.01$) of the extract activity at either storage procedure (−20°C and liquid nitrogen). This effect was only evident after one year of storage. Both sorbitol and glycerol gave rise to a final activity of about 130% in the extract stored in liquid nitrogen, while a significant difference ($P < 0.01$) was apparent between them in samples stored at −20°C; sorbitol achieved a higher protective effect (80% final activity) than glycerol (about 55%).

Regarding cathepsin D (Fig. 3-C), besides the significant difference ($P < 0.01$) between samples without cryoprotectants described above, both sorbitol and glycerol gave rise to a highly significant increase of the extract activity from one year of storage onwards. Indeed, their presence resulted in an increase in final activities from 80% to 120% when the extract was stored at −20°C and from 100% to 130–140% when it was stored in liquid nitrogen.

The BSLE extract activity on myofibrils is depicted in Fig. 3-D. Both cryoprotectants exerted a quite similar protective effect to that exerted on cathepsin D. Final values of proteolytic activity were significantly ($P < 0.01$) increased from 70% to 100% when the extract was stored at −20°C, and from 80% to 110% when it was stored in liquid nitrogen.

4. Discussion

The main problem associated to the industrial application of enzyme extracts, besides its preparation in large amounts, concerns its loss of activity throughout storage, despite crude extracts are known to retain enzyme activities more easily and longer than purified enzymes. Melendo et al. (1998a) [3] reported the enzymic characterization of a bovine spleen lysosomal-enriched (BSLE) extract. This extract showed a high proteolytic potential for its use in the tenderization of muscle foods, while maintaining a highly desirable eating quality (Melendo et al., 1998b, 1998c) [7,8]. Those results proved the technological suitability and usefulness of its use. Thus far, to our knowledge, no attempt had been made to investigate the preservation of the proteolytic activity of a spleen lysosomal-enriched extract. In our present work, the BSLE extract, possessing a pH value of 5.8, has been subjected to preservation by storage under different conditions: refrigeration at 0°C in an ice bath for 60 days; freezing at −20°C —either directly or previously frozen in liquid nitrogen—, −80°C and in liquid nitrogen; freeze-drying and stored at 0°C; and freezing at −20°C or in liquid nitrogen in the presence of glycerol or sorbitol as cryoprotectants. A particular attention was paid to the effective proteolytic activity of the extract on its foreseeable industrial target substrate, that is myofibrillar proteins as a whole.

Storage under refrigeration at 0°C demonstrated its usefulness for preserving a major part of some of the enzyme activities, namely cathepsin D, exopeptidase and the proteolytic activity on myofibrils, for a limited period of 60 days. However it was ineffective for preserving activity of cathepsins B, B+L and H, as well as proteolytic activity on purified myosin. These results agreed in part with those reported by Kirschke and Barrett (1987) [21] and Beynon and Bond (1989) [22] for purified enzymes. Besides this, our results suggest that the effective proteolytic activity on myofibrils is not related to cathepsins B, B+L or H activities, while it could well be related to cathepsin D action, since they show a high similarity in the rate of activity loss. Furthermore, effective proteolytic activity on myosin might be related to cathepsins B or L, since their rate of activity loss are quite similar.

Freezing of the extract resulted, as expected, in a longer preservation of enzyme activities, reaching in many cases up to 2 years. The lower the temperature of storage, the higher were the activities found, showing maxima at −80°C and in liquid nitrogen, in agreement with previous reports of Kirschke and Barrett (1987) [21] and Beynon and Bond (1989) [22]. The enzymatic activity of the BSLE extract was even increased above 100% of the initial activity. We have thus far no explanation for this effect, although it might be related to changes in the protein structure brought about by freezing conditions, which may lead to enhanced activity of enzymes (Sahagian and Goff, 1996) [23]. Again, the highest preservation ability was shown by cathepsin D and the effective proteolytic activity on myofibrils, which were both maintained even at a temperature of −20°C. This fact reinforces the suggestion that the responsibility of myofibril proteolysis other than myosin alone ought to be related to cathepsin D rather than to cathepsin B, L or H. Freeze-drying demonstrated its ability to keep cathepsin activity at a high level, while it failed to preserve the effective activity on myofibrils.

The ability of sorbitol and glycerol for protecting the enzyme activities of the BSLE extract from a loss of activity has been undoubtedly demonstrated. Regarding the effective activity on myofibrils, the use of both cryoprotectants assured the preservation of initial activity for a period of up to 2 years, even at a freezing temperature of −20°C.

As a consequence, the BSLE extract may be considered as a suitable tool for proteolytic treatments of muscle foods, since its enzyme activity is easily preserved for at least 2 years. The most useful conditions appear to be either freezing at temperatures of −80°C or below, or freezing at −20°C in the presence of cryoprotectants.

5. Conclusion

The effective proteolytic activity on myofibrils of a lysosomal-enriched extract, prepared by simple differential centrifugation from a bovine spleen homogenate, was maintained at a high rate for two years by freezing at −80°C or in liquid nitrogen. Cathepsin D showed lower activity losses than cathepsins B and B+L at any storage condition. Preservation of both cathepsin activity and effective proteolytic activity on myofibrils was enhanced by using sorbitol or glycerol as cryoprotectants at any freezing temperature, including −20°C.

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