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A Pig Collagen Peptide Fraction. A Unique Material for Maintaining Biological Activity During Lyophilization and During Storage in the Liquid State

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Abstract

There is frequent use of human and animal proteins as stabilizers during lyophilization of a variety of biological substances with a view to long term stable storage. This report describes the comparative excellent stabilizing effect of a porcine collagen peptide fraction (CPF) during the lyophilization and subsequent storage of three commonly used biological substances, alkaline phosphatase, tissue plasminogen activator and thrombin.

The CPF was heated to 150°C for one hour before use. The CPF was shown to have some advantage during lyophilized storage over human serum albumin. Solutions of thrombin stored in CPF at room temperature and at 37°C for one week retained nearly all activity, while storage of thrombin in human serum albumin solution at 37°C lost nearly all biological activity.

These preliminary data suggest that porcine CPF is a safe and advantageous stabilizer for addition to biological products with a view to long-term lyophilized storage and short-term liquid storage.

Maintenance of stability during handling and storage of purified proteins with assayable biological activities is of special importance for the production of standard preparations. Besides the avoidance of denaturing treatment, specific protein stabilizing procedures comprise the addition of either antioxidants or reducing agents to prevent oxidative degradation, of proteinase inhibitors to prevent proteolysis, of chelating agents to exclude heavy metal ions, or of bacteriostatics and fungistatics to prevent microbial growth. Loss of potency by physical actions such as adsorption, surface denaturation, heat denaturation, desiccation, freezing and thawing, can be significantly reduced by the addition of glycerol, carbohydrates, amino acids, hydrophilic polymers or, inert proteins.

Human serum albumin (HSA), bovine serum albumin (BSA) and ovalbumin are commonly used as stabilizing agents and bulking material for protein freeze-drying. While both BSA and HSA carry minimal risk of viral and mycoplasmal contamination, BSA may, in addition, be a carrier of bovine spongiform encephalopathy (BSE) (Kimberlin 1991). The BSE agent has been characterized as a prion that withstands current sterilizing procedures (Wilesmith et al 1991). A further possible disadvantage of HSA and BSA as stabilizing agents may relate to their purity and possible contamination with biologically active materials such as proteinases or proteinase inhibitors which may interact with the biological activity being stabilized (Lipinski 1995; Gubler et al 1993). For some applications, heat-induced gelification and coagulation of albumin may cause additional problems. Moreover, albumin is usually added to the relevant biological substance in such amounts that it is not possible to examine the lyophilized substance by physicochemical procedures.

Gelatin, prepared from bones and skins of slaughtered animals and representing a degradation product of collagen and ossein, has also been used as a stabilizing agent for biologically active proteins. However, the property of gelatin to form gels or highly viscous solutions can affect the use of filter membranes and restrict its practical application. Depending on the source and purification technology, gelatin may contain relatively high amounts of heavy metal ions and other inorganic and organic contaminants which may damage the active proteins.

While the above caveats relating to various additives to biologically active materials have been known for many years, the recent anxiety surrounding the use of stabilizing proteins of human and bovine source has stimulated a more aggressive search for alternatives. Other excipients used in lyophilization are mannitol, sucrose and trehalose.

An alternative stabilizing agent is a polypeptide fraction of purified porcine skin collagen (Prionex, Prostab, Pentapharm Ltd), denoted henceforth as collagen peptide fraction, CPF. This report describes its preparation and assessment as a stabilizing agent.

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Materials and Methods

Preparation of porcine collagen peptide fraction (CPF)

Type I collagen from pig skin was purified according to the procedure of Light (1985). Freshly frozen pig skin was ground and defatted by solvent extraction. The resulting skin fibre pulp was treated with pepsin to solubilize type I collagen. While insoluble material was removed by filtration, collagen was precipitated from the filtrate at pH 7·5, dissolved in saline and further purified by salt fractionation and ion-exchange. Precipitated type I collagen was suspended in water and the pH adjusted to 3·5 with hydrochloric acid. The acidified suspension was heated in an autoclave for 60 min at 145°C while the concentration was adjusted with water to $10 \pm 1\%$ solids. This solution was filtered under aseptic conditions through a 0·2-mm membrane to yield a sterile, 10% CPF solution with an

endotoxin content below 10 endotoxin units mL^{-1} and a heavy metal content below 20 ppm. As estimated by analytical gel chromatography, the average molecular weight of CPF solution is 20 kDa with a range of 5–45 kDa.

Preparation of recombinant tissue plasminogen activator (rt-PA) bulk material

Clinical grade recombinant freeze-dried t-PA (from Boehringer-Ingelheim, Germany) was used in this study. A vial of 50 mg rt-PA was reconstituted as instructed by the manufacturer with sterile distilled water, divided into 1-mL portions, flash frozen and stored at −40°C. The t-PA when reconstituted in this way contained 200 mM arginine in buffer. The frozen samples contained 620×10^3 int. units using the International Standard for t-PA (Gaffney & Curtis 1987) for comparison in a clot lysis assay system.

Preparation of thrombin bulk material

Purified α-thrombin was kindly supplied by Dr J. Fenton (New York Health Authority, Albany, NY, USA) and was stored in 1-mL portions containing 2·9 mg mL^{-1} α-thrombin. Each portion contained 10×10^3 int. units thrombin activity using the International Standard for α-thrombin (Gaffney et al 1992) as a calibrator, in a clotting assay procedure.

Preparation of thrombin, tissue plasminogen activator and alkaline phosphatase for lyophilization

Thrombin for lyophilization and storage. Samples of α-thrombin were thawed and sufficient volume was added to each of four different diluents to give 300 mL of solution with a presumed potency of 100 int. units mL^{-1}. These four diluents were 0·5 and 1% w/v HSA solution in 0·01 M phosphate buffer (pH 7·4) and 0·5 and 1% w/v CPF solutions in the same buffer.

t-PA for lyophilization and storage. Samples of t-PA were thawed and sufficient volume added to each of the four diluents described to give 300 mL each of a solution having a presumed potency of 1000 int. units mL^{-1}.

Alkaline phosphatase for lyophilization and storage. Affinity-purified alkaline phosphatase, derived from bovine intestinal mucosa was purchased from Sigma Chemical Co. (Poole, Dorset, UK) and has been described elsewhere (Ford & Dawson 1993). It contained 10 000 units per vial and was dissolved in 0·1 M Tris buffer (pH 7·2) to a solution of 12·9 units mL^{-1} with either no additive or containing 0·5 or 1% w/v CPF or 0·5 or 1% w/v HSA.

Lyophilization of alkaline phosphatase, thrombin and tissue plasminogen activator solutions

Each of the samples described above was dispersed in 1-mL volumes into DIN type 5-mL ampoules at 4°C. The ampoules were placed on an Edwards Lyomax 5 freeze-drier shelf, precooled to −50°C, and the contents frozen at the rate of approximately 1°C min^{-1}. When all the samples were frozen to −50°C some ampoules of each preparation were removed, sealed, labelled 'frozen baseline' and stored at −150°C. The remainder were freeze-dried followed by further desiccation according to the method employed to prepare biological reference materials (Campbell 1974) and as further modified (WHO **1988**).

Stability tests

Ampoules from each lyophilized preparation were placed at temperatures of −20, 4, 20, 37, 45 and 56°C for periods of up to 12 weeks before testing for activity. Solutions of thrombin and tissue plasminogen activator were stored at 4 and 25 and 37°C for 7 days and the loss of activity was observed as a percentage of the same solutions snap-frozen and stored at −70°C.

Assay of thrombin

This was essentially a clotting assay performed on the automated ACL-300 (Instrumentation Laboratories, Warrington, Cheshire, UK) or using the KC-4A coagulometer (Amelung, Germany) both of which have been described elsewhere (Gaffney & Edgell 1995).

Assay of t-PA

This was essentially performed by the method of Verheijen et al (1982) using a cyanogen bromide-activated fibrinogen (CNBR-FG) as a promoter in the assay.

Assay of alkaline phosphatase

This was essentially performed as will be described elsewhere.

Results

Fig. 1 shows histographic presentation of the biological activities of ampouled t-PA, thrombin and alkaline phosphatase following storage at various temperatures for 12 weeks. Since the concentration of both HSA and CPF from which lyophilization took place did not seem to affect the degradation profile (data not shown) only comparison of 0·5% solutions is shown. The samples stored at −20°C were nominated a potency of 1·0 or 100% and all the stored potencies are related to this 1·0 or 100%. While there was significant thrombin degradation (~20%) following storage at 56°C for 12 weeks there was no difference between the CPF (0·5%) and the HSA (0·5%) data. The data for t-PA degradation following lyophilization in CPF (0·5%) and HSA (0·5%) shows that only at 56°C storage did the lyophilized t-PA show a significant loss of activity in 12 weeks using both additives. The degradation data for alkaline phosphatase show that there was no difference in the stabilizing effect of CPF and HSA during storage of the lyophilized alkaline phosphatase. While there was significant loss in activity at all storage temperatures there was a statistically insignificant indication that lyophilization from a 0·5% CPF solution was advantageous at storage temperatures of 45 and 56°C. Thrombin solutions (100 int. units in buffered 0·5% HSA and 0·5% CPF) and t-PA solutions (1000 int. units in buffered 0·5% HSA and 0·5% CPF) were stored at 4, 20 and 37°C for 7 days. Both t-PA solutions showed no loss in activity at 4 and 20°C while there was a 30% loss of activity in the 0·5% HSA solution at 37°C as against no significant loss in the 0·5% CPF. Thrombin solution in 0·5% HSA showed nearly total loss of activity when stored for 7 days at 37°C and 66% loss at 20°C while there was no detectable loss of thrombin activity when storage was in 0·5% CPF solution (Table 1).

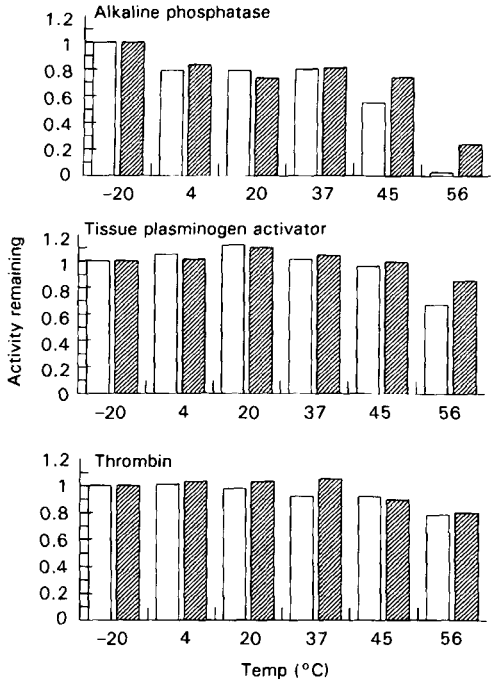

FIG. 1. Biological integrity of alkaline phosphatase, recombinant tissue-type plasminogen and human thrombin stored at various temperatures for 12 weeks. The stored materials were lyophilized from solutions of 0.5% human serum albumin (□) and 0.5% pig collagen peptide fraction (□).

Table 1. Biological integrity of thrombin solutions stored in 0·5% human serum albumin and 0·5% collagen peptide fraction solutions for seven days at various temperatures.

Temperature (°C)	Activity remaining	
	HSA (0·5%)	CPF (0·5%)
4	0·94 ± 0·01	0·93 ± 0·02
20	0·34 ± 0·09	0·90 ± 0·03
37	0·09 ● 0·01	0·89 ± 0·02

Discussion

In the area of standardization it is essential that a standard should freeze-dry well and maintain its activity for prolonged periods of storage. While it is unlikely that temperatures of 45 and 56°C occur during transportation of standards it is essential that standards should not lose significant amounts of activity when stored at 37°C for one month, this being the possible fate of a standard in transit to some countries in high summer. Heretofore, albumin (bovine or human) has proven adequate to stabilize small amounts of most biological activities during lyophilization and storage. The anxiety about proteins of both human and bovine origin has generated an interest in other stabilizing materials. The alternative used in this study, porcine collagen peptide fraction (CPF), is an acid hydrolysate of Type I porcine collagen and has been shown to be comparatively non-immunogenic in rabbits (data not shown).

The data shown above have indicated that using three different biological activities, (one was a commercially used thrombolytic agent, tissue plasminogen activator (t-PA), while the other two, thrombin and alkaline phosphatase, are laboratory used hydrolytic enzymes for which international standards exist), 0·5% CPF solution is as effective as human serum albumin for protecting biological activity during lyophilization and subsequent storage. Transport of materials without the addition of human or bovine proteins has advantages in terms of the presumed safety for handling staff during international transportation.

At a concentration of 0·5% CPF the cost difference with 0·5% HSA is minimal especially when filling large numbers of ampoules (> 1000), as is currently the practice in the preparation of national and international standards. Recent standards for single-chain urinary-type plasminogen activation were lyophilized in the presence of 0·5% CPF and were demonstrated as a single band on detergent polyacrylamide gel electrophoresis since the excipient CPF did not stain in this electrophoretic system. This is a major advantage for any standard since it allows the use of the standard for aspects of physicochemical comparison rather than exclusive biological activity which has been a limitation heretofore. While this study was aimed essentially at the preparation and transport of biologically active standards, our data also suggest that CPF solution may have a role to play as an additive to biologicals used in therapy. Our data (Table 1) indicated that biologically active solutions in CPF have enhanced stability in solution compared with the same activities solubilized in albumin solution. This suggests that biological activities which require slow infusion at room temperature or 37°C may benefit from reconstitution in CPF solutions. Safety and lack of immunogenicity are further added benefits.

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