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Protection of T4 bacteriophage against inactivation during freezing and thawing by addition of peptides

By P. R. M. STEELE
*University Department of Pathology, Cambridge, England**

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SUMMARY

Peptides of rabbit globin produced by tryptic digestion, were found to be highly protective against inactivation of freeze-thawed T4 phage. In concentrations of about 10^{-3} M the peptides protected the phage against inactivation by both concentrated NaBr in the unfrozen aqueous phase and the eutectic phase change.

Fractionation of the peptides by G25 Sephadex showed that peptide concentration rather than peptide size was the more important factor in determining the degree of protection of the phage by electrolyte effects. In contrast, protection against eutectic injury was strongly dependent on peptide size.

Possible mechanisms of action of the protective peptides are discussed.

INTRODUCTION

Peptone additives in concentrations of 5–10% (w/v) have been widely used as protectives against freeze-thawing and freeze-drying injury to micro-organisms and viruses, although the mechanism of the protective effect is unknown. Recently, Steele, Davies & Greaves (1969*a*) showed that separation of a 1% peptone solution on a G25 Sephadex column yielded some fractions which were highly protective for T4 bacteriophage against freezing injury. The protective compounds were believed to be small peptides. The peptone preparation being used ('Bacteriological Peptone' – Evans Ltd.) was an unspecific papaine digest of muscle and unsuitable for further investigations. What was required was a pure protein which could be split enzymically into peptides of known composition. The amino acid sequence and positions of tryptic cleavage of rabbit globin have been determined by Von Ehrenstein (1966) and Braunitzer, Best, Flamm & Schrank (1966), and the details of G25 Sephadex separation of the tryptic digest peptides have been published by Hunt, Hunter & Munro (1968). This paper reports the preliminary findings of using globin peptides as protective additives.

MATERIALS AND METHODS

The methods of phage preparation and purification, and the techniques used for freezing, thawing and titre determinations were the same as those described previously (Steele *et al.* 1969*a*, *b*). Percentage survivals were determined by plaque assay.

* Present address: Middlesex Hospital Medical School, Cleveland Street, London, W. 1.

Preparation of purified rabbit globin and tryptic digestion

The red cells of 30 ml. rabbit blood were washed six times in saline and then lysed in distilled water. The red cell membranes were removed by centrifugation at 15,000 *g* for 15 min. The haemoglobin solution was then dialysed against distilled water for three days at 4° C. The haem was removed by spraying the dialysed haemoglobin solution into acetone containing 2% (v/v) concentrated HCl at −20° C. with fast magnetic stirring (Rossi-Fanelli, Antonini & Caputo, 1958). The resulting white precipitate of globin was washed in acid-acetone, acetone, acetone-ether 50% (v/v) and ether (twice) and then dried in a current of air.

The globin was dissolved at 10 mg./ml. in distilled water + 2% trypsin (Bovine Pancreatic Trypsin – Sigma & Co., U.S.A.). The globin was precipitated by adding NH_4HCO_3 to a final concentration of 0·1 M, and digestion then carried out for 2 hr. at 37° C. The pH was maintained at 8·2 by addition of 0·1 M-NH_4OH. After digestion the solution was neutralized with acetic acid. Insoluble material was coagulated by freezing and thawing and then removed by centrifugation. The supernatant was freed from volatile salts by triple freeze-drying.

Sephadex fractionation of the globin tryptic digest

Hunt *et al.* (1968) analysed the peptide composition of the fractions obtained by G 25 (fine grade) Sephadex fractionation of globin tryptic digest (G.T.D.). They found that the fractions fell into eleven peaks whose position could be determined by measuring the O.D. at 280 mμ. 250 mg. of G.T.D. was fractionated through the Sephadex column of Hunt *et al.* (160 cm. long, 800 ml. bed-volume) using 1·0 M acetic acid as eluent. The O.D. at 280 mμ was continuously monitored during fractionation by a Uvicord LKB recorder (LKB Produkter, Stockholm, Sweden). The 10 ml. fractions were freeze-dried twice and redissolved in 2·5 ml. distilled water. The relative peptide concentration of the fractions was measured by O.D. at 215 mμ. The optical densities and peak positions are shown in Fig. 2A. Although the absolute concentration of the peptides was not measured, 250 mg. of digested globin fractionated into 2·5 ml. fractions is equivalent to a maximum concentration of 2×10^{-3} M for each peptide in any fraction.

RESULTS

Protective effect of globin tryptic digest.

Two test systems were used to determine the protective effect of the G.T.D. (i) Protection of the T4 phage against the denaturing effect of electrolytes concentrated during freezing was investigated by adding G.T.D. to T4 phage suspended in 0·1 M-NaBr. The samples were cooled at 1° C./min. to −17·5° C. and thawed slowly (to avoid osmotic injury) – control survival 1%. (ii) Protection against inactivation of the T4 phage at temperatures below the eutectic temperature of the suspending medium was investigated by adding G.T.D. to T4 phage suspended in 0·13 M phosphate buffer (KH_2PO_4-Na_2HPO_4, pH 7). The samples were cooled at 1° C./min. to −45° C. and then thawed slowly – control survival 11%.

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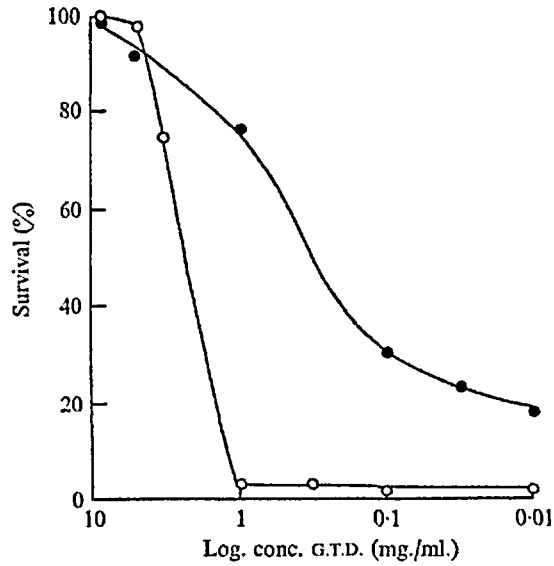

Fig. 1. Protection of T4 phage with globin tryptic digest (G.T.D.) against ionic (○) or eutectic (●) injury during freezing and thawing.

The protective action of different concentrations of G.T.D. against the two types of inactivation are shown in Fig. 1. The graph shows a sigmoid relationship between the G.T.D. concentration and protection of the T4 phage against inactivation below the eutectic temperature ('eutectic injury'), whereas the protective effect against the inactivation by electrolyte concentration ('ionic injury') is curiously discontinuous.

Inhibition of protection by added amino acids

Earlier work with peptone fractions (Steele *et al.* 1969*a*) had shown that fractionation of peptone actually increased the protective action of some of the fractions above that observed for whole unfractionated peptone. The removal of inhibitory compounds by fractionation was suggested as the explanation for this effect. The report also showed that amino acids had no protective effect by themselves and might have been the inhibitors removed from the peptides by fractionation. To test this possibility, pure amino acids were added in concentrations of 0·01 M to T4 phage samples suspended in phosphate buffer containing 5 mg./ml. added G.T.D. The samples were cooled at 1° C./min. to −45° C. and then thawed slowly. The added G.T.D. was omitted in a control series of samples. The results are shown in Table 1. The added amino acids had no significant effect of their own, with the exception of phenylalanine which lowered survival, but all of them considerably inhibited the protective action of the G.T.D.

Protective effect of Sephadex fractions

The Sephadex fractions were tested for protective effect against ionic injury and eutectic injury in the same way as was used to test the whole digest (Figs. 2B, C).

Many of the fractions were highly protective against ionic injury, the degree of

Table 1. *The inhibitory effect of amino acids when added to T4 phage frozen with 5 mg./ml. added globin tryptic digest* (G.T.D.). *The samples were cooled at 1° C./min. to −45° C. and thawed slowly*

	Survival (%)	
Added amino acid 0·01 M	0·13 M buffer	0·13 M buffer + 5 mg./ml. G.T.D.
None (control)	12	90
Glycine	8	51
Alanine	11	29
Valine	8	20
Leucine	4	18
Iso-leucine	6	18
Serine	11	49
Threonine	13	27
Methionine	6	18
Phenylalanine	0·6	18
Tryptophane	10	35
Histidine	11	37
Arginine	14	33
Lysine	24	36
Aspartic acid	12	35
Glutamic acid	12	35
Proline	—	19

protection appearing to depend on the relative peptide concentration (O.D. 215 mμ) rather than on the peptide size (fraction number).

In contrast the protection against eutectic injury increased with increasing fraction number, indicating that smaller peptides gave greater protection. The peaks of protection also matched fairly well with the positions of the peptide peaks. Table 1 showed that unprotective amino acids blocked the effect of the larger protective peptide molecules. By analogy it is possible that small peptides exert a dominant action over larger peptides. On the basis of this consideration a plot was made, using the data of Hunt *et al.* (1968), of the smallest peptide of each peak (as measured in amino acid units) against the maximal survival associated with each peak (Fig. 3). This showed a possible correlation with peptide length and indicated that maximal protection was associated with di- or tri-peptides.

Protection by dilysine and trilysine

The peptide bonds split by tryptic digestion have a C-terminal basic peptide of arginine or lysine. Since Fig. 3 indicated that di- or tri-peptides might give the best protection against inactivation of T4 phage by eutectic injury, a tryptic digest of polylysine was prepared as this results in an equal mixture (by weight) of dilysine and trilysine (Waley & Watson, 1953).

Polylysine with an average molecular weight of 175,000 (Sigma & Co., U.S.A.) was dissolved at 10 mg./ml. in 0·1 M-NH_4HCO_3 and digested for 2 hr. at 26° C. with 0·2 mg./ml. added trypsin. The resulting digest was freeze-dried twice. Paper chromatography confirmed that it contained roughly equal proportions of dilysine and trilysine but was essentially free from lysine.

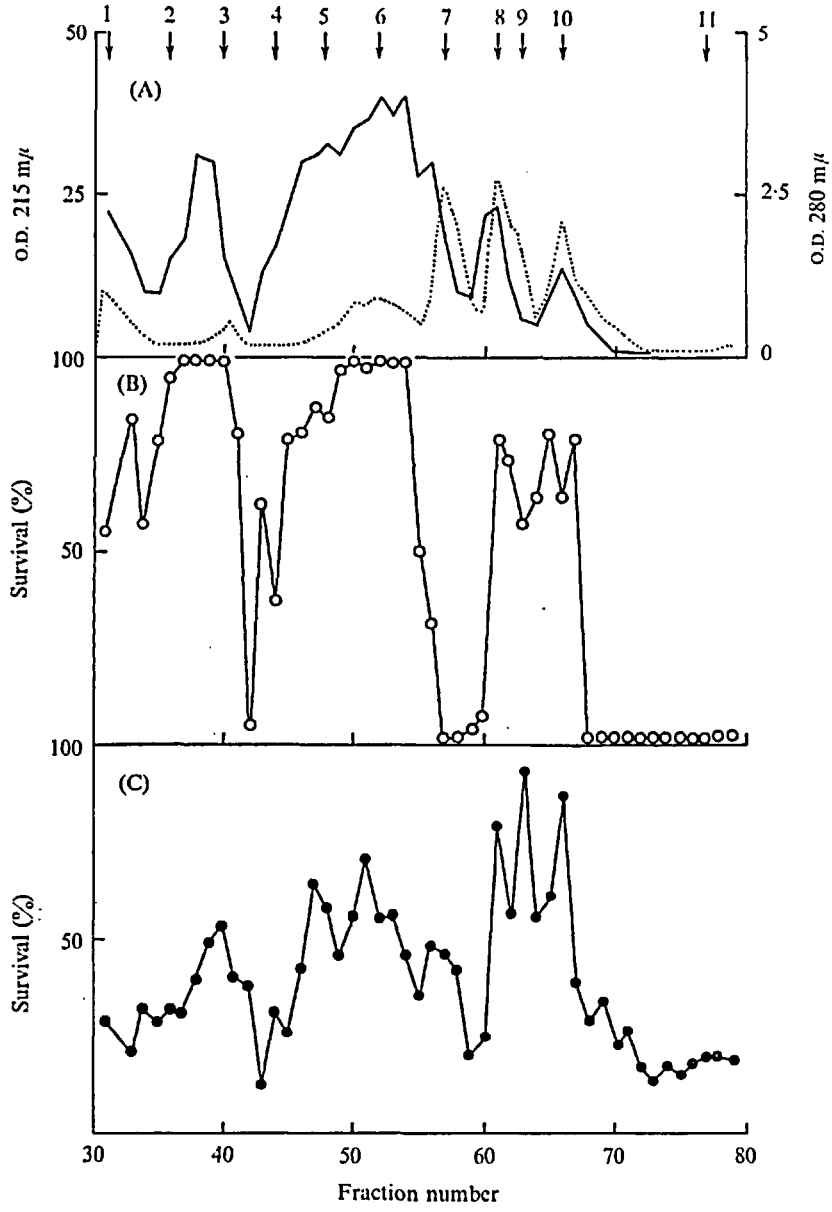

Fig. 2. The protective effect of G 25 Sephadex fractions of globin tryptic digest on the viability of freeze-thawed T 4 phage.

(A) The optical densities of the fractions at 280 mμ (dotted line) and 215 mμ (solid line). The positions of the peptide peaks analysed by Hunt *et al.* (1968) are also shown.

(B) Protection against ionic injury. Samples of T4 phage suspended in 0·1 M-NaBr + fraction were cooled at 1° C./min. to − 17·5° C. and then thawed slowly. The control survival (no added fraction) was 1 %.

(C) Protection against eutectic injury. Samples of T4 phage suspended in 0·13 M phosphate buffer + fraction were cooled at 1° C./min. to − 45° C. and then thawed slowly. The control survival (no added fraction) was 11 %.

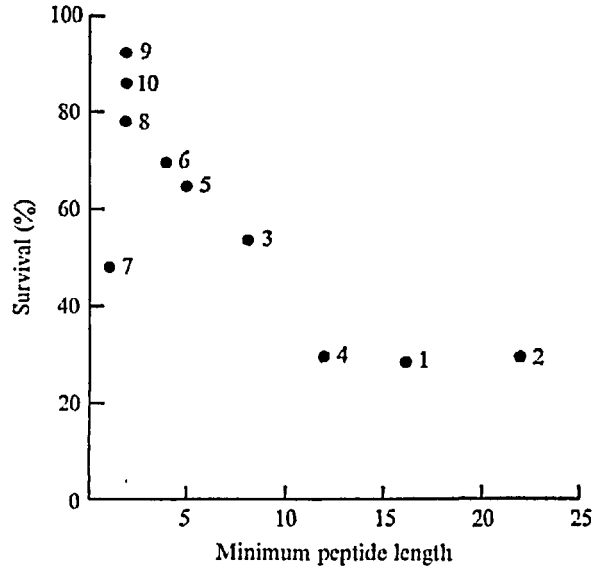

Fig. 3. A possible correlation between peptide length (in amino acid units) and protection of T4 phage against eutectic injury during freezing and thawing. The peaks of protection of Fig. 2 (C) are shown as a function of the shortest peptide present in the respective fraction (data from Hunt *et al.* 1968).

The digest was added at 1 mg./ml. (equivalent to $1{\cdot}5 \times 10^{-3}$ trilysine + 2×10^{-3} dilysine) to T4 phage suspended in 0·13 M phosphate buffer. Samples were cooled at 1° C./min. to −45° C. and then thawed slowly. There was 100% survival of these protected samples compared with the control survival of 11% in samples in which the polylysine digest was omitted.

The digest was also tested for protective activity against ionic injury: 1 mg/ml. of the digest was added to T4 phage suspended in 0·1 M-NaBr. Samples were cooled at 1° C./min. to −17·5° C. and then thawed slowly. There was 62% survival of these protected samples compared with the control (no added digest) survival of 1%.

Undigested polylysine at a concentration of 2 mg./ml. gave no protection against ionic or eutectic injury, when tested in the same way as the tryptic digest.

DISCUSSION

The mechanism by which strong solutions of electrolytes denature proteins is still a matter of controversy. The degrees of denaturation produced by different salts is highly dependent on the species of ions, and in the present context the mechanism of inactivation of T4 phage by high electrolyte concentrations is called 'ionic injury' for simplicity and convenience. The protective effect of peptides against ionic injury was extremely powerful, and their mode of action is therefore of considerable interest. If ionic injury is due to the effects of ions on the solvent properties of water, especially in regard to the hydrophobic interactions which stabilize the native protein configurations (Von Hippel & Schleich, 1969), it is difficult to believe that such low concentrations of added peptides (10^{-3} M)

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could neutralize the effect of a 100-fold greater concentration of NaBr in the test system. This leaves the alternative explanation that the peptides protected through an interaction with the phage proteins, which would at first seem improbable since no correlation was observed between peptide size and protective effect. However, it is well known that molecules strongly bound to proteins (e.g. substrate added to an enzyme) give good protection against denaturation. Tanford (1968) shows that even one molecule strongly bound to a protein molecule might decrease the rate of denaturation by a factor of 1000-fold. Hence peptide molecules which bind to phage proteins could protect against denaturation whatever their length.

In contrast, the results indicated that the protection afforded by peptides against eutectic injury was dependent on peptide size (as measured by amino acid units and Sephadex filtration), smaller peptides (e.g. dilysine and trilysine) giving the best protection. When the eutectic phase transition occurs, the suspended T4 phage particles are suddenly transferred from a liquid phase to a solid phase and inactivation results ('eutectic injury'). The mechanism of eutectic injury is unknown but perhaps it is caused by removal as ice of water essential for the native protein configurations. On such a hypothesis the protective peptides could block the removal of protein-bound water from labile sites by an interaction with the protein, or alternatively they might stabilize the proteins against conformational alterations even if some essential structural water were removed. Such a hypothesis would explain the dependence on peptide size since only those peptides which bind to the labile sites could be protective. Also peptides which protect the phage against eutectic injury should protect against ionic injury as well, although the converse would not be true. This consideration is in fact well shown in Fig. 2.

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