Techniques for the Fractionation of Microbiologically Active Peptides Derived from Casein

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Hearfield & Phillips (1961) reported that the rate of growth of a strain of *Streptococcus equisimilis* in a medium based on acid-hydrolysed casein was markedly enhanced by the addition of a small amount of an enzymic digest of casein.

It seemed desirable to investigate more fully the complex mixture of peptides in such digests of casein and to establish the respective contributions made to the biological activity of digests by individual peptides.

In the past, investigations of this type were hampered by the extreme difficulty in separating peptides, first from amino acids and secondly from one another. This paper contains a description of techniques which have been successfully applied to the resolution of the peptides of casein digests and, in addition, some results on the distribution of growth-stimulating activity among the peptide fractions.

EXPERIMENTAL

Materials. Calcium caseinate was supplied by Glaxo Laboratories Ltd., acid-hydrolysed casein by Oxo Ltd., 'slab yeast' by The Distillers Co. Ltd., Sephadex G-25 by Pharmacia, Uppsala, Sweden, and Hyflo Supercel by Johns Manville and Co. Ltd.

Organism. The test organism, which was a strain of *Streptococcus equisimilis* (CN 2688 in The Wellcome Research Laboratories' Culture Collection) was freeze-dried and stored in ampoules until required.

Crude ox trypsin. This was prepared by the method of Cole & Onslow (1916). The solution obtained by this means had a content of total solids of about 3% (w/v). From its method of preparation the product almost certainly contained chymotrypsin and certain peptidases in addition to trypsin. For simplicity of expression, however, digests of casein prepared by the use of this extract will be referred to as 'trypsinized casein'.

Yeast extract. An aqueous suspension of 'slab yeast' (25%, w/v) was boiled for 30 min. Cell debris was removed by filtration with kieselguhr as filter-aid and the filtrate was then sterilized by passing it through a Ford SB pad. The resulting extract had a content of total nitrogen of about 0.1% (w/v).

Inoculum medium. The inoculum medium was of the following composition: yeast extract, 2.5 ml.; acid-hydrolysed casein, 2.25 g.; L-cystine, 1.5 mg.; DL-tryptophan, 1.0 mg.; sodium bicarbonate, 0.2 g.; thioglycollic acid, 0.05 ml.; glucose, 0.2 g.; cold tap water to 100 ml. After adjustment to pH 7.6 with 10N-sodium hydroxide the medium was sterilized by filtration.

Assay medium. The assay medium differed from the inoculum medium in containing 12.25 ml. of yeast extract, 3.0 g. of glucose and in addition 1.25 ml. of a solution (0.1%) of phenol red.

'Trypsinized casein'. Trypsinized casein was prepared by a modification of the method of Gladstone & Fildes (1940). An aqueous slurry containing calcium caseinate (10%, w/v) and sodium carbonate (0.37%) was stirred in a closed container for 4 hr. Crude ox trypsin (0.6%) and chloroform (about 1%) were then added and the temperature of the digest was raised to 37°. The pH value of the mixture, which fell during the early stages of digestion, was adjusted to 7.5 at intervals of 20 min. by the addition of 10N-sodium hydroxide. The digestion was allowed to continue at 37° with constant stirring for a total of 72 hr. After boiling for 10 min., insoluble material was removed by filtration, Hyflo Supercel being used as filter aid. The filtrate was autoclaved at 15 lb./in.² for 20 min. and stored at 0° until required. The digest had a content of total nitrogen of about 1.3% (w/v) and a content of total solids of about 10% (w/v).

Preliminary fractionation of casein digests. A glass tube (250 mm. × 39 mm.), fitted at its lower end with a porous plate, was packed with a slurry of Sephadex G-25 (50 g.) in 0.1M-phosphate buffer, pH 6.8. The exact composition and even the pH of this buffer seemed to be of no special significance. As the manufacturers suggested, however, even dispersions of Sephadex were most readily produced in solutions containing inorganic salts. After the column had been allowed to settle, the top surface was covered with a disk of filter paper and the phosphate buffer displaced from the column with aq. 0.2M-ammonia solution.

The sample (2.5 ml.) of trypsinized casein was applied to the top surface of the column, allowed to soak in and then washed through with aq. 0.2M-ammonia solution. The effluent from the column was collected automatically in fractions (40 × 8 ml.) at a rate of 10 fractions/hr. Ammonia solution was used in this procedure as it is readily removed from fractions and hence does not interfere with subsequent treatment of separated materials.

Location of peptides in fractions from columns of Sephadex. In preliminary experiments, peptides and free amino acids were detected by treatment with ninhydrin after chromatographic separation on paper with butan-1-ol-acetic acid-water (Woiwod, 1949) as solvent. Subsequently it was found more convenient to examine column effluents by the following two methods.

(a) By determining the extinction of each fraction at 280 mμ. This indicated the distribution among the various fractions of free and combined aromatic amino acids, and yielded quickly and easily a permanent record of the column run. This was useful for comparison of different digests and for checking the reproducibility of columns.

(b) In the procedure to be described, the content of total amino acid in each fraction was determined colorimetrically after hydrolysis with alkali. A portion (0.025 ml.) of each fraction was pipetted into water (1.0 ml.) in a test tube and 7.5N-sodium hydroxide solution (0.3 ml.) was added. The mixture was heated at 100° for 2.5 hr. and, after cooling, 5N-acetic acid (1.0 ml.) was added. The following reagents prepared as described by Yemm & Cocking (1955) were then added in the order given: 0.2M-citrate buffer, pH 5.0 (0.5 ml.), 5% solution of ninhydrin in 2-methoxy-ethanol (0.2 ml.), 0.2M-potassium cyanide in 2-methoxy-ethanol (1.0 ml.). After mixing, the solutions were heated at 100° for 15 min. and cooled for a further 15 min. in running tap water.

The resultant purple solutions were diluted with aq. 60% ethanol (to 10 ml.) and the extinction of each solution was compared with that of a reagent blank with a Hilger Biochem absorptiometer and a filter with peak transmission at about 580 m μ . By reference to a calibration curve prepared with glycine, the results were expressed as total amino acid (mm).

Analysis of fractions from Sephadex columns by 'finger-printing'. Peptide fractions from column runs were evaporated to dryness *in vacuo* and weighed. The solid was then dissolved in sufficient water to make a solution containing 250 mg. of solid/ml. A portion of this solution was applied to a sheet of Whatman 3 mm paper marked out as for two-dimensional chromatography. When a paper was loaded with a solution at the concentration referred to above, the end of a capillary containing the sample was applied to the paper and the liquid allowed to run out until a spot of diameter 1 cm. was formed. The spot was dried in warm air and a further application of sample made to give a spot of the same size. After drying, the sample was fractionated by descending chromatography with butan-1-ol-acetic acid-water (75 ml./sheet) as solvent. After 24 hr. the sheet was allowed to dry overnight at room temperature and the edge of the paper which had been in the chromatographic trough was trimmed off to yield a sheet of width 39 cm. The paper was then laid on a clean bench with the line occupied by the partially separated peptides along a glass rod of diameter about 2 cm. and buffer solution allowed to flow from a large hypodermic syringe on to the paper first on one side of the sample line and then on the other. It was convenient to make this application of buffer at a distance of 1 cm. from each side of the sample line. After the two buffer edges had met along the whole length of the sample line, the remainder of the paper was dipped into the buffer and blotted, care being taken not to blot the sample.

The sheet was then hung in a Perspex tank, a simplified form of the apparatus described by Katz, Dreyer & Anfinsen (1959), with the sample line horizontal and at the lower end of the paper. The upper end of the paper passed over a glass rod into a Perspex trough containing buffer solution, and the lower end of the paper dipped into a layer of buffer solution at the bottom of the tank. To help in dissipating the heat arising from the electrophoresis, the apparatus was filled completely with colourless domestic-grade paraffin. A potential difference of 2500 v was then applied to the paper by means of a platinum anode, in the trough of buffer, and a platinum cathode, in the buffer layer at the bottom of the tank. To minimize the effects of changes in pH in the vicinity of the cathode, it was en-

closed in a cellophan bag. With phosphate buffer, pH 7.7, 1.0-0.5 (Green, 1933), duration of the electrophoretic separation was 1 hr. In this time, the current rose from about 100 to 130 ma and the temperature of the paraffin at the top of the tank rose 15–20°. At the end of the run, the paper was removed from the tank, dried at 110° and sprayed with a solution of ninhydrin and collidine in chloroform (Woiwod, 1960). On heating at 110° for about 10 min. many peptide spots became visible. Further spots developed on storing the paper overnight in polythene bags at about 4°.

Estimation of growth-stimulating activity of peptide fractions. The contents of an ampoule of dried culture were suspended in nutrient broth and streaked on blood-agar slopes. After incubating overnight at 37° a loopful of organisms was transferred from a slope into the inoculum medium (10 ml.) and kept at 37° for 8 hr. A loopful of the resulting culture was transferred into fresh inoculum medium (10 ml.), which was kept at room temperature for 9 hr., then heated to 37° in a water bath and maintained at that temperature ($\pm 0.05^\circ$) for a further 7 hr. Portions (0.1 ml.) of the culture were then used to inoculate the test media. The latter comprised portions (10 ml.) of the assay medium in 150 mm. $\times$ 15 mm. test tubes to which peptide fractions had been added. As controls, it was customary to inoculate both the basal assay medium and the assay medium fortified with 0.5% (w/v) of trypsinized casein.

Measurements of growth were made at intervals of 30 min. by determining the extinction of each culture in a Hilger Biochem absorptiometer with a filter with peak transmission at 680 m μ . Acid produced by the organism was neutralized with 0.2N-sodium hydroxide immediately before these measurements.

RESULTS

Fractionation of casein digests on columns of Sephadex. Filtration of digests through Sephadex led to a fractionation of the type illustrated in Fig. 1. Thus four groups of components were detected by the ninhydrin colorimetric procedure whereas three groups of substances were detected by measurements of ultraviolet absorption. Examination of the fractions by paper chromatography showed that tubes 10–14 contained complex material, as was evident from its streaky behaviour on chromatograms and feeble reaction with ninhydrin.

Tubes 15–20 contained peptides which chromatographed fairly well, and tubes 21–32 contained simple amino acids. Aspartic acid and glutamic acid were somewhat anomalous in their behaviour as they emerged at about tube 18.

The aromatic amino acids phenylalanine, tryptophan and tyrosine, which were very much retarded on the column, formed a peak at tube 27.

The pattern of fractionation was reproducible with a single batch of Sephadex. On comparison of batches, however, some variation was noticed, particularly in the degree of separation achieved between free amino acids and peptides of intermediate size.

Tests for growth-stimulating activity. The technique used for the preparation of the inoculum for the tests led to the early onset of rapid growth. Thus it was normally necessary to make additions of alkali within the first 3.0–3.5 hr. after inoculation, and an increase in extinction was often detectable within 1 hr. The rate of growth of the organism after 6–7 hr. in a medium containing 0.5% of trypsinized casein was about five times as great as that in the basal medium alone (Fig. 2).

When groups of fractions (pooled as shown in Fig. 1; i–vi) from a column of Sephadex were tested, only the first four groups were found to be significantly active. The activity of the first fractions from the column was rather low but rose quickly to a maximum value in a region corresponding to the second ninhydrin-positive fraction. Thereafter, the activity fell away sharply to a low value in the fractions containing amino acids (cf. Fig. 1).

Separation of fractions of casein digests by 'finger-printing'. When the finger-printing procedure was applied to unfractionated digests of casein, only amino acids were detected, as individual peptides

were not present at sufficiently high concentrations to be revealed by the ninhydrin reagent. The amino acids which were found were: leucine/isoleucine, tryptophan, phenylalanine, valine, methionine, tyrosine, proline, alanine, serine, glycine, threonine, aspartic acid and glutamic acid. Lysine, histidine and arginine appeared to have migrated into the buffer solution at the cathode end of the paper. As the procedure was not directly applicable to digests of casein, individual fractions from columns of Sephadex were examined. It was then found that whereas the peptides in fractions 9–14 were too complex to separate well in the buffer system employed, the intermediate peptides in fractions 15–20 were extremely well resolved.

The groups of fractions (i–vi) from Sephadex, referred to above, when subjected to the finger-printing procedure, gave rise to patterns, examples of which are illustrated in Fig. 3. As each fraction gave a characteristic pattern, it was clear that the

Fig. 1. Fractionation of a casein digest by filtration through Sephadex G-25. Distribution of constituents was determined by measurement of extinction at 280 mμ (O) and by the colorimetric ninhydrin procedure (●). The activities of pooled fractions (i–vi) are shown by the block figure. The values for activities were obtained from $A = (E_T - E_0)/E_0$, where E_0 and E_T are respectively extinctions of cultures grown in the basal assay medium and in the assay medium to which one-quarter of each of the pooled fractions had been added (1:4). Values of A at 6, 7 and 8 hr. growth were averaged.

Fig. 2. Growth curves of *Streptococcus equisimilis* in assay medium containing: (a) 0.5% of casein digest; (b) one-quarter of pooled fraction (iii); (c) one-quarter of pooled fraction (i); (d) no addition. Extinction readings were corrected for changes in volume of culture fluid arising through titration of acid formed during growth.

treatment with Sephadex had resulted in an effective separation of peptides.

The buffer referred to in the Experimental section appeared to possess a satisfactory combination of properties. Thus the ionic strength

selected did not lead either to excessive heating on the one hand, or to very marked endosmotic flow on the other. Similarly, the pH value selected appeared well suited to the separation of all but the most basic compounds.

Fig. 3. Separation of peptides in fractions from Sephadex by finger-printing. (a) Fraction (iii). (b) Fraction (iv). (c) Fraction (v). (d) Fraction (vi). Key: (1) leucine/isoleucine; (2) tryptophan; (3) phenylalanine; (4) valine/methionine; (5) tyrosine; (6) proline; (7) alanine; (8) threonine; (9) glycine; (10) probably serine; (11) aspartic acid; (12) glutamic acid.

DISCUSSION

The use of paper chromatography and high-voltage electrophoresis, successively, clearly leads to the resolution of complex mixtures of peptides to a degree not possible by methods previously employed. Indeed, so detailed and reproducible are the patterns obtained that Ingram (1958) and Katz *et al.* (1959) have been able to use the technique to detect very fine differences in detailed structure between pairs of closely related proteins. It was, of course, in this context that the term 'finger-print' was first applied to the patterns obtained.

It was not feasible with trypsinized casein to use the above-mentioned method directly, owing to the low concentration of individual peptides relative to that of the free amino acids. The digest was therefore subjected to a preliminary fractionation of a type which would separate peptides from free amino acids. Such a fractionation appeared impossible by classical procedures, but recent reports by Porath (1960), that compounds of differing molecular weights may be separated from one another by filtration through the cross-linked dextran Sephadex, offered much promise. It was found that the filtration of digests through Sephadex G-25 not only gave good separation of peptides from amino acids, but the elution patterns suggested that some separation of peptides into groups had taken place. The possibility of using eluting agents other than aqueous ammonia solution was not investigated in detail, but it seems probable from observations by Porath (1960) on the separation of amino acids, peptides and proteins on columns of Sephadex that the selection of eluting agent is not critical.

Aqueous ammonia solution was convenient to use in the present instance as it conferred upon the system some measure of control of pH, was readily removed from fractions by vacuum distillation at low temperature and in addition served as a preservative when it was necessary for fractions to be stored. The presence of ammonia in the fractions did not lead to excessively high blanks in the ninhydrin colorimetric method, probably owing to the loss of ammonia which occurred during the alkaline-hydrolysis stage.

By comparison with haemoglobin, which is said to be completely excluded from the Sephadex matrix, it was concluded that the largest molecules in digests of casein were similarly completely excluded and hence passed through the column unretarded.

The aromatic amino acids, on the other hand, remained on the column until a volume of eluent had passed through which corresponded to the internal volume (V_i) of the Sephadex. The value of

V_i for the column was calculated from the product of the weight of Sephadex (g.) and the water-regain quoted by the manufacturers.

The value of the preliminary fractionation on Sephadex was immediately evident, as digests when treated in this way and subjected to the finger-printing procedure gave satisfactory patterns of separation. Finger-prints of individual fractions from the column in many instances showed differing patterns of peptides, confirming the impression formed earlier that the use of Sephadex led to separation of certain peptides from one another as well as from free amino acids. Whereas both of the methods referred to have separately been described by other authors, the present work, in which over one hundred different peptides have been detected in trypsinized casein, indicates that maximum usefulness may be derived from these methods by applying them successively.

Turning to the more detailed examination of fractions obtained from Sephadex, the observation that four well-defined peaks were detected by the ninhydrin method, whereas only three components were detected by measurements at 280 m μ , suggests that, in the digest, aromatic amino acids occur in combination in relatively few molecular species; the failure of the two sets of curves to show any correspondence indicates that these compounds constitute only a small proportion of the total peptide material. The observation that the bulk of the growth-stimulating activity was located in a fairly narrow region of the total effluent suggests that maximal activity is associated with peptides of particular molecular dimensions. In this connexion, Merrifield & Woolley (1958), working with *Lactobacillus casei*, failed to detect high streptogenin activity in peptides containing fewer than five amino acid residues and showed that an upper limit existed for the size of peptides with this type of activity.

Hearfield & Phillips (1961) have described the separation from trypsinized casein of factors which appeared to exert their action at different stages in the growth of the bacterium. In the present study no such effect was detected and, indeed, the fractions from Sephadex gave rise to a series of growth curves which were similar to one another, differing only in their slopes. As the fractionation on Sephadex may be regarded as a form of molecular sieving, it may be inferred that the two types of growth pattern previously referred to do not, contrary to expectation, reflect the action of peptides differing on the basis of molecular weight. Indeed, it now seems likely that fractions of a given mean molecular weight contain a balanced mixture of peptides active in both the early and later stages of growth. It therefore appears probable that the previously reported differences in bio-

logical properties of peptides derived from casein reflected more specific differences in chemical constitution.

SUMMARY

1. Columns of Sephadex G-25 have been used for the preliminary fractionation of tryptic digests of casein.

2. Fractions from Sephadex columns have been examined for their ability to stimulate the growth of a strain of *Streptococcus equisimilis*. Maximum stimulatory activity appeared to be localized in a fairly narrow region of the total peptide material.

3. Further fractionation of peptides derived from casein has been achieved by subjecting fractions from columns of Sephadex to two-dimensional separations involving the successive use of paper chromatography and high-voltage electrophoresis. Over one hundred spots due to peptides have been detected.

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The Colorimetric Reaction between Vitamin A₂ Aldehyde and Antimony Trichloride

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The occurrence of vitamin A₁ aldehyde in the eggs of some marine teleosts has recently been reported (Plack, Kon & Thompson, 1959). To extend this study to the eggs of freshwater teleosts, a method for the determination of vitamin A₂ aldehyde was required, since preliminary experiments had shown that the A₂ form was present together with vitamin A₁ aldehyde in salmon eggs (Plack, 1958). Cama *et al.* (1952) and Farrar, Hamlet, Henbest & Jones (1952) have prepared crystalline vitamin A₂ aldehyde and, for the colorimetric reaction with antimony trichloride, gave $\lambda_{\max}$ 730–740 m μ fading to 705 m μ , and 730 m μ fading to 705 m μ , respectively. Wald (1938–39) had previously found this $\lambda_{\max}$ to be 705 m μ , and Wilt (1959), with extracts from natural materials, found $\lambda_{\max}$ to be between 700 and 705 m μ . The study of the colorimetric reaction between vitamin A₂ aldehyde and antimony trichloride reported here shows how these differences of $\lambda_{\max}$ may have arisen and, though not exhaustive, provides the basis for a quantitative test.

EXPERIMENTAL

Preparation of vitamin A₁ aldehyde. Vitamin A₁ aldehyde was prepared from the crystalline alcohol (Roche Products Ltd.) as described by Plack *et al.* (1959). For the preparation of vitamin A₂ aldehyde, the A₁ aldehyde was purified by chromatography but not crystallized.

Preparation of vitamin A₂ aldehyde. Vitamin A₁ aldehyde (2.38 g.) was converted into vitamin A₂ aldehyde as described by Henbest, Jones & Owen (1955). The crude preparation in light petroleum (b.p. 40–60°), was adsorbed on a 20 cm. × 4 cm. column of 300 g. of aluminium oxide (British Drug Houses Ltd., for chromatographic adsorption analysis) weakened by the addition of 30 ml. of water, and the column was developed with 2.5 l. of 2% (v/v) of freshly-redistilled diethyl ether in light petroleum (b.p. 40–60°). A yellow diffuse band was eluted first, followed by a strong orange band, but neither contained vitamin A₂ aldehyde. The main orange band was eluted more slowly and was collected in three fractions. From their absorption curves, fraction 1 contained much vitamin A₁ aldehyde and was rejected, but fractions 2 and 3 contained reasonably pure vitamin A₂ aldehyde and were combined; the solvent was removed under reduced pressure, and the residue dissolved in 30 ml. of light petroleum (b.p. 40–60°). Spontaneous crystallization occurred overnight at –20°, appar-

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