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NOTES

Identification of a Peptide from Mammal Albumins Responsible for Enhanced Pigment Production by Group B Streptococci

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The peptide from peptones responsible for enhanced pigment production by *Streptococcus agalactiae* in culture media has been isolated from a peptic digest of human albumin and has been identified as Ile-Ala-Arg-Arg-His-Pro-Tyr-Phe. The related heptapeptide lacking the N-terminal Ile also had pigment-enhancing activity. A sequence similarity search showed that these sequences are present only in mammal albumins.

Streptococcus agalactiae (group B streptococcus [GBS]) is an important cause of infections in newborns and adults (18, 21). Human beta-hemolytic GBS produce an orange or red pigment that can be detected on certain culture media (4, 8). This method is very specific and can be used to detect and identify GBS (1, 9a). Peptone (8), starch (5, 8, 13, 20), folate pathway inhibitors (3), and serum (8) are important components of culture media for pigment detection of GBS. Some peptones, such as proteose peptone (PP) no. 3 (Difco, Detroit, Mich.), have pigment-enhancing (PE) activity, and pigment production is poor when other peptones are used (3, 8, 12, 20). The serum component that accounts for its PE activity has been identified as amylase (15). However, the active component of peptones had not been defined, and because this knowledge could help improve the design of culture media (19), we undertook this study to identify it. PE activity was detected by bioautography (9, 14, 15). Two beta-hemolytic GBS strains were used; one was isolated from a patient and identified by accepted procedures (9a, 16), and the other was *S. agalactiae* ATCC 13813. The test medium was New Granada Medium (4) in which PP from Oxoid (Basingstoke, United Kingdom) was substituted for PP no. 3. This medium also contained serum, starch, methotrexate, glucose, and a morpholinepropanesulfonic acid (MOPS)-phosphate buffer. Previous experiments had shown that in this medium GBS pigment production was poor. Twelve milliliters of medium (at 50°C) was poured into 9.5-cm-diameter petri dishes, mixed with 20 μl of an overnight culture of the test strain in brain heart infusion broth, and left to solidify. In each plate, 7-mm-diameter wells were cut and filled with 40 μl of each dilution of the fluid to be assayed in distilled water. The plates were incubated under anaerobic conditions (85% N_2, 10% H_2, and 5% CO_2) for 18 h at 37°C, and a zone of orange-red GBS microcolonies formed around the wells, showing PE activity. A unit of PE activity (PEU) was defined as the activity present in the well with the highest dilution of each biological fluid that showed activity. Protein levels were determined by the bicinchoninic acid procedure (kit from Pierce Biochemicals, Rockford, Ill.). The peptide concentration was determined by measuring the absorbance at either 280 or 215 nm (model 220S spectrophotometer; Hitachi, Tokyo, Japan). All chromatographic separations were done with a Pharmacia (Uppsala, Sweden) system (FPLC Controller LCC System 500 Plus). The protein concentration in eluates was monitored by measuring the absorbance at either 280 or 214 nm (Uvicord II apparatus; Pharmacia). Chromatographic columns were also from Pharmacia. Tris-Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (17) was run in a Mini-Protean II cell (Bio-Rad Laboratories, Hercules, Calif.). Gels were stained with Coomassie brilliant blue. Molecular weights were estimated with markers from Bio-Rad. Mass spectra were acquired in a Bruker Biflex MALDI-TOF spectrometer by using 3,5-dimethoxy-4-hydroxycinnamic acid as an ionizing matrix. Protein sequencing was carried out in a Procise microsequencer (Perkin-Elmer–Applied Biosystems). Peptide synthesis was carried out by following the manufacturer's protocols in a Synergy 432A 9-fluorenylmethoxycarbonyl synthesizer (Perkin-Elmer).

PE activity was detected in the Difco peptones PP no. 3 (0.001 PEU/μg), PP no. 2 (0.0005 PEU/μg), PP (0.0005 PEU/μg), and Peptamine (0.001 PEU/μg); in the Sheffield peptone (Quest, Norwich, N.Y.) Primatone RL (0.0005 PEU/μg); and in a peptone prepared by hydrolyzing human serum with pepsin. However, activity was not detected in any of the Difco products peptone, tryptone, tryptose, and Soytone; in the Oxoid products PP, Lab Lemco, and lactoalbumin hydrolysate; in the Sheffield products HY Soy, Primatone HS, N-Z Amine A, N-Z Amine E, N-Z Amine HD, and Amisoy N-Z; or in peptones prepared by hydrolyzing human serum with trypsin, ficin, or proteinase K.

When a PP no. 3 solution was ultrafiltered by using a membrane with a molecular mass cutoff of 1,000 Da (Millipore Co., Bedford, Mass.) activity could be recovered from the ultrafiltrate. However, this ultrafiltrate lost its activity when hydrolyzed with trypsin. We hypothesized that the active substance was a peptide. Owing to the difficulty of characterization of active compounds in peptones (2, 19), we attempted to hydrolyze a protein of known sequence, where activity could be detected,

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and to identify the active peptide. We tested enzymatic digests (pepsin, trypsin, proteinase K, and ficin) of several proteins (human and bovine albumin, ovalbumin, gamma globulin, cytochrome *c*, hemoglobin, myoglobin, thyroglobulin, insulin, β-lactoglobulin, aprotinin, actin, α2-macroglobulin, ferritin, and α-amylase from hog and human saliva). Activity was found only in peptic digests from human and bovine albumin (0.04 PEU/μg). As the sequence of albumin is perfectly known (11), we tried to identify the PE products from pepsin-treated fragments of this protein. Hydrolysates were made with hog pepsin (0.05 mg/mg of protein) at 50°C for 4 h, after the pH was adjusted to 2.5 with HCl.

One gram of human albumin (99%, fatty acid and globulin free; Sigma Chemical Co., St. Louis, Mo.) was cleaved with CNBr (6), and the three fragments obtained (10) were separated in two steps: (i) size exclusion chromatography in a column (95 by 2.6 cm) of Sephacryl HR 100 (Pharmacia) with 50 mM formic acid and (ii) application of the fraction with the lowest molecular weight (two peptides) from step 1 to a 6-ml column of a strong cationic exchanger (Resource S; Pharmacia). One peptide was eluted with 25 mM phosphate buffer (pH 6.7) and the other peptide was then eluted with 0.5 M NaCl. This second peptide is a 175-aa peptide (from Cys124 to Met298) (10). After pepsin hydrolysis, all the activity was in this 175-aa peptide (0.1 PEU/μg). This peptide was cleaved at its tryptophan (Try215) with dimethyl sulfoxide-CNBr (7). The products were dissolved in 6 M guanidine HCl–0.6 M Tris HCl (pH 8.5)–100 mM 2-mercaptoethanol and were separated (the yield was about 50%) by size exclusion chromatography in a column of Superdex 75 eluted with the same buffer. The two peptides (91 and 84 aa) from the cleavage of the 175-aa peptide were separated from each other by reversed-phase chromatography with a Source 15 column equilibrated with 0.1% trifluoroacetic acid (TFA) and eluted with a linear gradient to 50% CH_3CN–0.1% TFA. After pepsin hydrolysis of each of these two separated peptides the activity was in the 91-aa peptide (from Cys124 to Trp214) (0.2 PEU/μg). This peptide was hydrolyzed with immobilized pepsin (Sigma). Afterward, the products from this hydrolysis were separated by reversed-phase chromatography with a PEP 5/5 column equilibrated with 0.1% TFA and eluted with a linear gradient to 17% CH_3CN–0.1% TFA. Activity was detected only in a single peak that was resolved in four peaks by size exclusion chromatography in a Superdex peptide column eluted with 20% CH_3CN–0.1% TFA. Activity was detected only in the first of these peaks (0.5 PEU/μg). Sequencing showed that this peak was a mixture of two peptides: Ile-Ala-Arg-Arg-His-Pro-Tyr-Phe (albumin positions 142 to 149) and Phe-Ala-Lys-Arg-Tyr-Lis-Ala-Ala-Phe (albumin positions 157 to 165).

These peptides were synthesized, and only Ile-Ala-Arg-Arg-His-Pro-Tyr-Phe was active (2 PEU/μg). However, the activity of this peptide was lost when the peptide was hydrolyzed with trypsin. Afterward, the peptides Ala-Arg-Arg-His-Pro-Tyr-Phe, Ile-Ala-Arg-Arg-His-Pro-Tyr, Ala-Arg-Arg-His-Pro-Tyr, Ile-Ala-Arg-Arg-His-Pro, and Arg-Arg-His-Pro-Tyr-Phe were synthesized; only the heptapeptide Ala-Arg-Arg-His-Pro-Tyr-Phe showed activity (2 PEU/μg). A search in the molecular biology server of the Swiss Institute of Bioinformatic (13a) showed that the amino acid pattern Ala-Arg-Arg-His-Pro-Tyr-Phe matched only with mammal albumins and with the albumin-derived peptide kinetensin.

This suggests that some peptones show PE activity because they are peptic digests of protein mixtures that contain albumin and so contain the peptide Ile-Ala-Arg-Arg-His-Pro-Tyr-Phe. This would account for the different abilities of commercial peptones to support the growth of GBS as pigmented colonies. In addition, this finding would also allow minimization of the variability in pigment production associated with the different types of peptones used in culture media for the detection of GBS pigment.

How these peptides cause their PE activity is not clear, and further investigation is necessary. Investigating specific activities in very complex mixtures of biological compounds, such as peptones, can be a very difficult task. However, the approach outlined in this work of using a defined starting product that leads to defined end products could be worthwhile in trying to solve similar problems.

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