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ISOLATION, AMINO ACID COMPOSITION, AND BIOLOGICAL ACTION OF A PEPTIDE BIOREGULATOR FROM COW K-CASEIN

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Research workers are currently paying great attention to peptide bioregulators, for these molecules occupy a central position in the system of endocrine regulation of the body. Not only substances traditionally regarded as peptide regulators, but also degradation products of unspecialized precursor proteins, may also exhibit biological activity.

It has been shown [5, 7, 8-10, 12] that the mammalian dietary protein, milk casein, during proteolysis forms a series of physiologically active peptides, capable of regulating the circulation and the secretory and motor functions of the stomach and of exhibiting opium-like activity.

Considering the extraordinary importance of peptide bioregulators as potentially useful therapeutic agents, it was decided to isolate a peptide preparation from k-casein with a distinct biological action and to study its amino-acid composition and physiological activity.

EXPERIMENTAL METHOD

Total casein was obtained from fresh, defatted milk by acid precipitation at the isoelectric point. α-Casein was isolated from total casein by Little's method and purified twice to remove traces of α_s- and β-caseins by precipitation from 50% ethanol with ammonium acetate [16], and dialyzed against 0.5% NaCl. A small fraction (10-20 ml) of the solution obtained after dialysis was freeze-dried and the purity of the α-casein was verified by electrophoresis. The purified α-casein was used to isolate the peptide. For this purpose, the concentration of α-casein was determined spectrophotometrically in the solution obtained after dialysis, on the assumption that $E^{0.1\%}_{280\,nm} = 1.120$, and it was diluted to a 2% concentration with 0.5% NaCl solution, heated to 37°C, after which the pH was adjusted to 5.65 with 0.1 N HCl, and a weighed sample of pepsin, dissolved in a small quantity of distilled water was added (at the rate of one part of enzyme to 100 parts of α-casein by weight). After incubation (2.5 min) a 50%

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solution of TCA was added to a final concentration of 12%, equivalent to 31.5 ml of 50% TCA to 100 ml of hydrolysate. The residue of protein and large polypeptides was left to form for 1 h, separated by filtration through filter paper, and discarded, and the filtrate was freed from TCA by extraction with equal volumes of diethyl ether with shaking for 3 min. The ether was changed 5 or 6 times and the aqueous fraction freeze-dried. The resulting peptide preparation, in an amount of 100 mg in 2 ml of 0.01 M ammonia solution, was fractionated on a column with Sephadex G-50 medium (1.5 × 105 cm), equilibrated with 0.01 M ammonia solution at the rate of 24 ml/h. The yield of the material from the column was recorded on the REPPS-1M apparatus at a wavelength of 260 nm and recorded automatically with the KSP-4 automatic writer. Fractions thus obtained were freeze-dried. Fraction 3 was the end product — a peptide with marked physiological action. The yield of protein was 100 mg from 10 liters of defatted milk.

The amino-acid composition was determined on a "Beckman 121" amino acid analyzer after hydrolysis in 6 N HCl in an atmosphere of nitrogen at 106°C for 24 h (the amino-acid analysis was done by Candidate of Medical Sciences A. P. Ekimovskii).

The action of the fractions on the CNS was estimated from the change in electrical activity of some deep brain formations and cortical regions, and also from the ability to depress the heart and respiration rates. Experiments were carried out on four rabbits with electrodes implanted in the above-mentioned structures. There were 12 experiments altogether. The rabbits were used in the experiments after starvation for 48 h. The electrical activity was recorded on an electroencephalograph from "Alvar-Electronic." After spontaneous electrical activity had been recorded the peptide was injected subcutaneously in a dose of 50 μg/kg. After 1 h, during the period of maximal manifestation of the physiological action of the peptide, naloxone (Endo Laboratories, USA), a blocker of opiate receptors, was injected subcutaneously in a dose of 75 μg/kg. The EEG continued to be recorded for 4-5 h after injection of the peptide.

EXPERIMENTAL RESULTS

After brief treatment of the k-casein with pepsin, precipitation of protein and large peptides with 12% TCA, purification of the supernatant from TCA by extraction with ether, and dreeze-drying of the purified extract, the original peptide material was obtained.

By gel-chromatography on a column with Sephadex G-50 the original peptide material was separated in four distinct fractions (Fig. 1). The first three fractions were collected and tested for their ability to alter the bioelectrical activity of the various brain structures. Fraction 3 was found to be a physiologically active peptide and, if infected subcutaneously into rabbits in a dose of 50 μg/kg, it induced single high-voltage (250 μV) waves with a frequency of up to 20 Hz (Fig. 2) after only 5-7 min. This effect was observed for 1.5 sec and was followed by a ν rhythm. On further recording it was found that these waves (16-20 Hz/ 250-350 μV) increased in duration to 105 sec, and on their replacement no σ-rhythm was present. The frequency of their appearance rose five-sixfold, and this effect lasted 4-5 h. The respiration rate was almost halved, and the heart rate reduced by 15-30%. Injection of the opiate receptor blocker, naloxone, abolished the effect of the peptide in 10-15 min.

The amino acid composition of the isolated peptide (fraction 3) is shown in Table 1, in which for comparison the composition of para-k-casein and of GMP, the N- and C-terminal parts of the k-casein molecule respectively, also is shown.

The amino acid profile of the isolated peptide contains residues of tyrosine, phenylalanine, histidine, and arginine, which are not present in GMP, but which are present in the para-k-casein moiety of the molecule, but the content of the threonine, valine, isoleucine, and leucine residues, which are present in both parts of the k-casein molecule, but in clearly different quantities, is similar to their content in para- -casein. These facts are evidence that the isolated peptide is a fragment of the para-k-casein moiety of the molecule.

Peptide bioregulators arising as a result of proteolysis of unspecialized precursor proteins usually perform their regulatory function within the sphere of the basic functions of the precursor protein. According to theoretical considerations [9], milk casein is a natural dietary protein of mammals.

The alimentary specificity of k-casein is manifested as the phenomenon of curdling of milk in the stomach, when rupture of one peptide bond [phenylalanine (105)-methionine (106)], which has enhanced sensitivity to the action of proteases, in the k-casein molecule leads to destruction of the micellar structure of the casein complex and to curdling of casein. Curd-

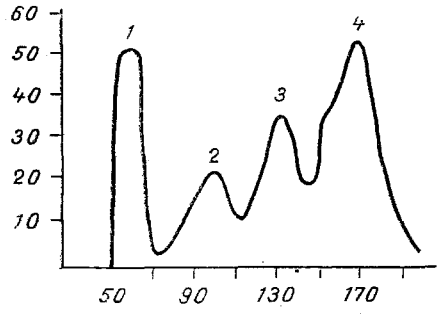

Fig. 1

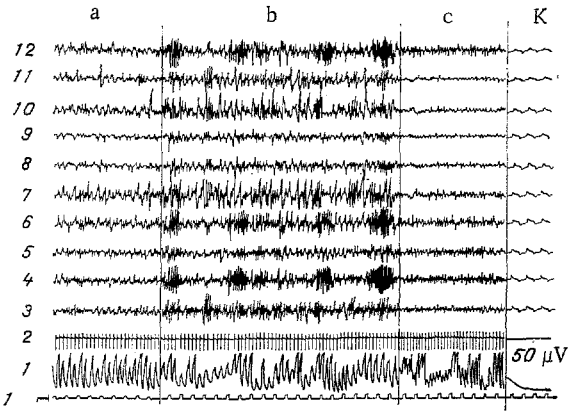

Fig. 2

Fig. 1. Chromatogram of TCA-soluble product of limited proteolysis of k-casein by pepsin. Abscissa, elution volume of substance from column (in ml); ordinate, optical density at 260 nm (% absorbance). Fraction 3 is a peptide with neurotropic action.

Fig. 2. Effect of neurotropic peptide on electrical activity of brain structures: a) spontaneous activity; b) after injection of peptide; c) after injection of naloxone. K) Calibration: 50 μV. 1) Respiration; 2) ECG; 3) reticular formation; 4) amygdala; 5) hypothalamus; 6-9) temporal cortex; 10-11) occipital cortex; 12) sensomotor cortex.

TABLE 1. Amino Acid Composition (in g/100 g peptide), Calculated from Amino Acid Analysis

Amino acid	Peptide (fraction 3)	para-K-casein	GMP
Aspartic acid (+ amide)	6,53	6,56	6,86
Threonine	7,50	2,42	16,83
Serine	5,80	4,96	7,80
Glutamic acid (+ amide)	14,26	17,78	19,39
Proline	12,60	9,51	11,78
Glycine	3,95	0,46	0,84
Alanine	6,98	5,20	6,34
Hemicystine	нет	1,81	нет
Valine	4,75	4,06	8,83
Methionine	0,21	1,07	1,94
Isoleucine	4,16	5,60	12,00
Leucine	6,78	6,44	1,67
Tyrosine	0,78	11,95	нет
Phenylalanine	2,35	4,70	нет
Lysine	11,36	6,27	5,72
Histidine	8,11	3,30	нет
Arginine	4,60	6,40	нет
Tryptophan	—	1,49	нет

Legend. Data for para-k-casein and GMP calculated from their primary structure [14, 15].

ling actively affects the rate of gastric evacuation [6] and, consequently, activity of the whole of the digestive-transporting container. During curdling a peptide inhibiting gastric secretion [10] and movements [8] is formed from k-casein. Thus the alimentary properties of k-casein are directed toward active regulation of the activity of the gastrointestinal tract.

According to Anokhin's theory [1] feeding is controlled by the complex alimentary functional system. Despite its complexity, the alimentary response has its own specific encephalographic manifestation in the form of high-frequency bursts of electrical activity in certain brain structures [11]. Similar changes in neuronal activity was observed in the period of food satiety, during infusion of amino acids into the blood, and also after administration of cholecystokinin [3, 4], which plays a key role in the development of a feeling of satiety [14].

The fact that the isolated peptide causes changes in neuronal activity similar to the effect of cholecystokinin and food satiety suggests that it has a role in the mechanism of satiation. The fact that the isolated peptide induces an encephalographic picture of satiety and tranquility in animals exposed to a stress situation (hunger for 48 h, strict immobilization for several hours) suggests that its action can be regarded as antistressor.

Slowing of the respiration and heart rates, and the abolition (even though only for a short time) of the action of the peptide by naloxone, are evidence that the opiate system is involved in the effects of the peptide.

The isolated peptide bioregulator, which is a natural product of digestion of one of the most widely used food proteins (milk casein), may be a promising therapeutic agent for weakening the sensation of hunger during treatment with reducing diets and also, perhaps, as a tranquilizing, antistressor preparation.

The possible biological role of the peptide we have isolated in neonatal feeding is evidently directed toward regulation of milk consumption with a view to preventing possible overfeeding and the consequent overloading of both digestive and protein-synthesizing and excretory systems of the newborn infant.

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