End-Group Analysis of α_{s1}-Caseins A, B, and C

Three genetically determined variants of α_{s1}-casein have been prepared, and the purity of each has been verified by starch-gel–urea electrophoresis. The amino-terminal end group was determined qualitatively by two independent chemical methods. Arginine was the only amino-terminal amino acid revealed by each method for all three variants. The results of the action of carboxypeptidase A can be interpreted most simply to infer a leucyl-tryptophan carboxyl-terminal sequence for all three proteins. The kinetics of amino acid release by carboxypeptidase was similar for the three α_{s1}-caseins. A molecular weight of ~31,000 was determined from the carboxypeptidase data, assuming a single polypeptide chain. The results indicate that all amino acid differences among the variants must occur within the polypeptide chain, if the latter assumption is valid.

The α-casein fraction of cow's milk has been the subject of many chemical and physical investigations. From electrophoretic behavior, phosphorus content, and amino acid composition, it was concluded to be homogeneous. Mellon *et al.* (1) and Wissmann and Nitschmann (2) carried out amino-terminal end-group analysis on this material by dinitrophenylation. These investigators found arginine and lysine to be the major amino-terminal residues of α-casein.

Later work revealed that the α-casein complex was actually a mixture of another phosphoprotein, κ-casein (3), and a protein designated α_s-casein (4). This α_s, the calcium-sensitive component of α-casein, is the major protein fraction of the casein family. Manson (5), realizing that previous structure work had been carried out on mixtures of α_s- and κ-casein, undertook an investigation of the N-terminal structure of α_s-casein free of contaminants. His results showed that arginine was the only N-terminal amino acid and that the protein had a minimum molecular weight of 31,000.

[1] Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

More recent investigations (6, 7) with starch-gel–urea electrophoresis and polyacrylamide-gel electrophoresis for separation of casein components have demonstrated the genetic heterogeneity of α_s-casein. Three genetically controlled variants have been found. These have been called α_{s1}-A, α_{s1}-B, and α_{s1}-C in order of decreasing electrophoretic mobility (8). Waugh *et al.* (9) have reported isolation of an α_s-fraction which is designated $\alpha_{s1,2}$-casein. It is probable that this material corresponds to a heterozygous mixture of two of the α_{s1}-casein variants (BC) mentioned above. C-Terminal end group studies on $\alpha_{s1,2}$ apparently revealed three terminal amino acids: tryptophan, tyrosine, and leucine.

When the three α_{s1}-caseins became available in pure form, an investigation to determine the terminal amino acids of these proteins was undertaken to ascertain the similarity or difference at these positions. This paper reports the results of the identification of the amino and carboxyl terminal amino acids of α_{s1}-caseins A, B, and C.

EXPERIMENTAL

Materials. The individual α_{s1}-casein variants A, B, and C were obtained from individual cows

typed homozygous for these proteins (8). The α_s-fractions were obtained from acid-precipitated whole casein by the following method, which is described in detail by Thompson and Kiddy (8). The whole α-casein complex was precipitated at 2°C with 0.40 *M* $CaCl_2$. Following removal of calcium from the calcium-sensitive fraction (crude α_s-casein), the protein was further purified by precipitation of contaminants from a 50% ethanol-water solution and DEAE-cellulose chromatography in 3.3 *M* urea. The isolated α_{s1}-caseins migrated as single zones (regardless of protein concentration) in starch-gel–urea electrophoresis at pH 8.6 and showed no contamination. The heterozygotes (AB, AC, and BC), however, migrated as paired zones. In the ultracentrifuge at pH 7.0, $\mu = 0.20$ (aggregated) and at pH 12.0, $\mu = 0.19$ (monomers), all three α_s-caseins sedimented as symmetrical components free of any visible contamination. Additionally, each homozygote α_{s1}-casein was stabilized against precipitation by calcium ions when κ-casein had been added.

Carboxypeptidase A was obtained from Worthington Biochemical Corporation[2] as a water suspension of three-times recrystallized material. It was treated with diisopropyl fluorophosphate (DFP) prior to use.

Methods: Carboxyl Terminal Amino Acids. For the determination of the C-terminal amino acids, hydrolysis with carboxypeptidase A was employed. The α_{s1}-caseins A, B, and C were dissolved in water at pH 7.8. The reaction was run in an unbuffered system at 37° with a weight ratio of enzyme to casein of 1:100. One-ml aliquots of the digestion mixture were withdrawn at specific time intervals ranging from 15 minutes to 22 hours. The aliquots were precipitated with 0.5 ml of 20% trichloroacetic acid, centrifuged in the cold, and the supernatant solutions were decanted and frozen until analyzed. The amino acids released were identified and quantitated by the ion-exchange chromatographic procedure of Moore *et al.* (10) using a sample corresponding to 15.0 mg of protein for analysis.

Amino Terminal Amino Acids. The FDNB (2,4-dinitrofluorobenzene) method of Sanger was employed for the qualitative determination of the N-terminal amino acids of the α_{s1}-casein variants. The procedures of reaction with FDNB, hydrolysis, and identification of DNP (2,4-dinitrophenyl)-amino acids were essentially those outlined by Levy (11). The DNP-arginine released was also identified by the modified Sakaguchi reagent (11).

The Edman degradation as applied for paper strips by Fraenkel-Conrat *et al.* (11) and Schroeder *et al.* (12) was used to confirm the N-terminal amino acids of the proteins. In addition to the starch-iodide detection method (11), the chromatographic identifications of the phenylthiohydantoin (PTH) derivatives of the N-terminal amino acids were carried out on nonstarched paper with the Sakaguchi reagent as the color developing spray.

RESULTS

The results of the hydrolysis of α_{s1}-caseins A, B, and C, with carboxypeptidase A, are shown in Figs. 1–3. The same amino acids were released from all three variants with tryptophan liberated first, followed by leucine. At 22 hours, slight traces of tyrosine, phenylalanine, and valine also appeared. The fact that the amount of leucine released exceeds the tryptophan after about 5 hours suggests that a second leucyl residue is located very close to the end of the chain. The 22-hour values are consistent with a possible leu-leu-try C-terminal sequence. However, the exact position of the second leucine cannot be unequivocally assigned from the data obtained in this experiment. Assuming the complete liberation of the C-terminal tryptophan and calculating on the basis of a moisture content of 13%, molecular weights of 26,100 for A, 31,300 for B, and 31,300 for C have been calculated.

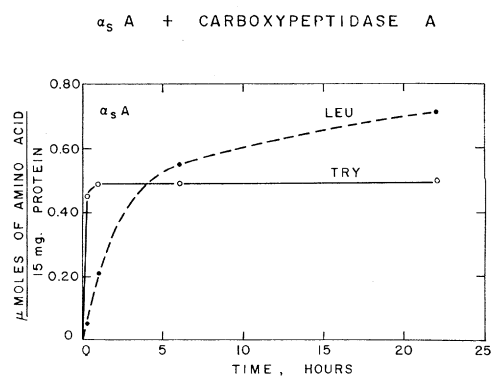

FIG. 1. Action of carboxypeptidase-A on α_{s1}-A casein. Weight ratio of enzyme to substrate, 1:100; pH 7.8 (unbuffered system); T = 37°C. Aliquot of digest equivalent to 13.0 mg of protein (moisture-free basis) analyzed at indicated times.

[2] It is not implied that the U. S. Department of Agriculture recommends the above company to the possible exclusion of others in the same business.

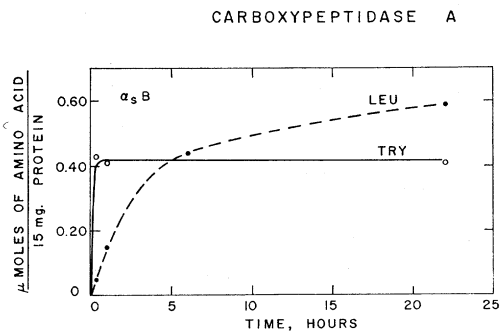

FIG. 2. Action of carboxypeptidase-A on α_{s1}-B casein. Weight ratio of enzyme to substrate, 1:100; pH 7.8 (unbuffered system); T = 37°C. Aliquot of digest equivalent to 13.0 mg of protein (moisture-free basis) analyzed at indicated times.

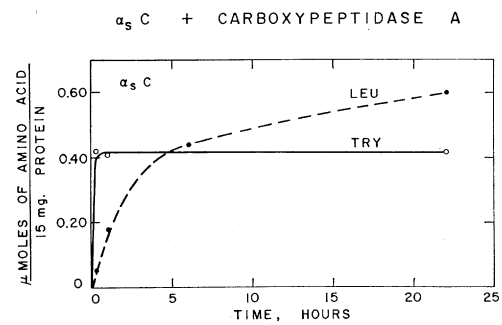

FIG. 3. Action of carboxypeptidase-A on α_{s1}-C casein. Weight ratio of enzyme to substrate, 1:100; pH 7.8 (unbuffered system); T = 37°C. Aliquot of digest equivalent to 13.0 mg of protein (moisture-free basis) analyzed at indicated times.

The FDNB procedure revealed only arginine as the N-terminal residue in all three of the α_{s1}-caseins. The ether-soluble fraction (11) of the hydrolysis mixture on chromatography contained only 2,4-dinitrophenol (DNP-OH) and 2,4-dinitroaniline in significant amounts. The DNP-OH was verified by reversible decolorization with acid. After chromatography of the water-soluble fraction (11), a yellow spot in the position of DNP-arginine was evident. To identify it positively as arginine and not ϵ-DNP-lysine, which has a similar mobility, the Sakaguchi reagent was again employed. This procedure gave a very strong orange spot for the control DNP-arginine and the DNP-unknowns at positions of exactly the same mobility. From these qualitative data, it may be concluded that arginine is at least one of the N-terminal amino acids of α_{s1}-caseins, A, B, and C. If a single chain is assumed, it would then follow that it is the only N-terminal amino acid.

The results of the Edman procedure with starch-iodide development were inconclusive. When the PTH of the N-terminal amino acid and PTH-arginine were chromatographed on nonstarched paper with solvent A (11), both gave positive tests with the Sakaguchi reagent at the position normally assigned to PTH-arginine. All three α_{s1}-caseins again gave the same result, which confirms the FDNB determination.

DISCUSSION

All three α_{s1}-casein variants appear to have the same amino and carboxyl terminal structure by the methods employed in the present study. The finding of only N-terminal arginine agrees with Manson (5) and Schmidt and Payens (14). The molecular weights, as calculated from tryptophan released by use of carboxypeptidase A, correspond well to the 31,000 calculated from arginine release, as reported by Manson. Also, the molecular weights of the variants, as determined from amino acid analyses, average to a value of 31,000 (13).

In addition, it is evident that all amino acid differences among the three variants, whether they be addition-deletion and/or substitution differences, must be located within the polypeptide chain, if the proteins consist of a single chain.

The results of the action of carboxypeptidase A on the α_{s1}-caseins differ somewhat from those presented by Waugh *et al.* (9), which indicate try, leu and tyr each as C-terminal amino acids of $\alpha_{s1,2}$-casein. The obvious question is the relation of $\alpha_{s1,2}$-casein, which gives a double band on starch-gel electrophoresis, to the homozygous α_{s1} variants. The conclusions of Waugh are drawn partially from the isolation of peptides from trypsin digests of amidinated $\alpha_{s1,2}$-caseins. Since all three of the amino acids in question are susceptible to the action of chymotrypsin, the possibility of contamination of the trypsin must be considered. Another point to be noted is the

cyanogen bromide treatment of the $\alpha_{s1,2}$-casein. The dialyzable tryptophan-containing peptide found may or may not be the C-terminal one. The nondialyzable material showing little tryptophan and considerable leucine may be incompletely split protein which has undergone destruction of the tryptophan under the conditions described. However, Waugh *et al.* (9) present some data for the action of carboxypeptidase A on the whole native protein and the whole amidinated protein which are not inconsistent with our results and could possibly be interpreted in sequential fashion rather than as three individual terminal groups.

However, the data presented in this report cannot rule out the possibility of two chains for each of the variants, especially in view of the 16,500 molecular weight value given by Schmidt and Payens (14). One chain would terminate in tryptophan which is rapidly released in comparison to the release of leucine from the second chain. However, the presence of a third chain terminating in tyrosine does not seem likely based on the data presented.

From this report it can be concluded that α_{s1}-caseins A, B, and C have identical N-terminal residues, namely arginine, and identical C-terminal sequences, leucine-tryptophan, if a single chain is assumed. Manson's data (5) also indicate a single chain of molecular weight $\sim$31,000 based on quantitative N-terminal arginine determinations. If a second chain is present, it could not terminate in arginine at the amino end if the molecular weight is actually 31,000. The chemical methods used in the present report would have failed to detect N-terminal cysteine, cystine, or half-cystine, but these amino acids are not found in α_{s1}-caseins (13). However, other amino acids such as tryptophan, histidine, or acylated amino acids would be difficult or impossible to detect by the methods employed. The purity of the individual caseins has been demonstrated by starch-gel–urea electrophoresis, which is the most discriminative tool available for studies of casein components. The molecular weights as determined by amino acid analysis agree with those found by end-group analysis. From amino acid composition data, the differences between the variants appear not to be single amino acid substitutions (13) and must be within the polypeptide chain.

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