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# TERMINAL AMINO ACIDS OF BUFFALO AND COW MILK CASEINS

*By*

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## SUMMARY

N-and C-terminal groups of acid precipitated caseins of buffalo and cow milk were quantitatively determined by paper chromatography using phenylation of casein and determination of the phenylated terminal amino acids and the hydrazine hydrolysis method respectively.

Results indicated that the buffalo and cow caseins contained the following respective averages of N-terminal residues expressed in mole per $10^5$ g of casein : 12.51 and 12.09 arginine, 2.06 and 1.59 lysine, 14.57 and 13.67 total N-terminal amino acids, 85 and 76 total free amino groups and 24.45 and 22.35 of chain lysine residue.

Also, both caseins contained the following C-terminal residues expressed in mole/$10^5$ casein : 2.24 and 2.46 glutamic acid, 3.94 and 3.58 aspartic acid, 2.18 and 2.20 glycine, 3.10 and 3.04 serine, 2.65 and 2.88 alanine respectively. Both caseins were found to contain the same average of 14.16 mole residue of total C-terminal amino acids per $10^5$ g of casein.

## INTRODUCTION

The terminal amino acids of proteins are partially responsible for their chemical and physical properties in addition to their biological specificity. The identification of these acids will probably provide a possible mean of testing the hyptothesis of the peptide arrangement in the protein molecule besides the differentiation between different proteins. These results will offer a great help to many industrial aspects in which casein plays a fundamental part. In addition, little work had been done on these acids in Casein particularly that of buffaloes. Therefore, this study is concerned with the investigation of the nature and the quantitative determination of the N-and C-terminal amino acids of buffalo milk casein and also of cows for comparison.

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## EXPERIMENTAL AND METHODS OF ANALYSIS

Bulk milk samples of both animals were obtained from the Faculty of Agriculture, Cairo University, and skimmed. Casein was precipitated at pH 4.6 by 1 N HCI, and centrifuged. The residue was washed thoroughly with distilled water, and dried with alcohol and ether.

*Identification and quantitative determination of N-terminal amino acids :*

Following the method described by Mellon, Conn and Hoover (1953), the N-terminal amino acids were determined by the alkaline phenylation of 0.2 g casein using dinitroflorobenzene(DNFB)at pH8 to give the dinitrophenyl-casein derivative (DNP-casein). The terminal DNP-amino acids in 0.1 g of DNP-casein were liberated by acid hydrolysis, extracted by ether until no more colour was found in the extract. The combined ether extracts were made up to 100 ml. The aqueous layer, was dried under vacuum at 60°-C, and then dissolved in acidified methyl ethyl ketone for paper electrophoresis.

The ether-soluble DNP-amino acids were identified by paper chromatography as described by Levy (1954). The chromatograms were developed for 24 hrs using toluene : pyridine : 2-chloroethanol : ammonia (35:9:18:18) as a solvent system, and di-DNP-lysine as the reference. Crystalline di-DNP-lysine was prepared from L-lysine by phenylation as reported by Sanger (1945). The quantitative determination of the ether soluble DNP-amino acids was carried out spectrophotometrically according to Biserte et al (1958), at 350 mu wavelength, using Beckman spectrophotometer, Model DU. The concentration was determined from a concentration curve of dinitrophenol in ether. The amount of di-DNP lysine was corrected putting in consideration that its recovery was 17%, (Mellon et al 1953).

The water-soluble DNP-amino acids were separated by paper electrophoresis according to Lockhart and Abraham (1954). Whatman No. 1 filter paper strips were used in borate buffer of pH 9.2 with ionic strength of 0.04 and 350 volt, and the electrophoresis run for 6-7 hrs. The quantitative determination was carried out spectrophotometrically from the electropherograms according to Biserte et al (1958), and Mellon et al (9531). The bands wer extracted with 1% sodium bicarbonate and the light absorption was read at 350 mu wavelength. The concentrations were calculated from a standard curve of dinitrophenol in 1% sodium bicarbonate, ranging from 0.1 to 0.8 micro mole. ε-DNP-lysine and DNP-arginine were used as references and corrected according to their recovery of 83% and 73% respectively.

The total DNP-amino groups of casein were determined spectrophotometrically at 350 mu wavelength using 0.1 N Nao H as a solvent (Biserte et al 1958). The concentration was determined from a standard solutions ranging from 0.1 to 0.8 micro mole in 100 ml of the alkali soultuion.

ε-DNP-lysine and a-mono-DNP arginine crystals were prepared from L-lysine and arginine respectively by phenylation using DNFB as reported by Sanger (1945).

*Identification and quantitative determination of C-terminal amino acids.*

Liberation of C-terminal amino acids were made according to Akabori and Natita (1952) by the hydrazinolysis of 0.05 g casein for 10 hrs at 100°C in a sealed tube. Their aqueous solution was purified by benzaldehyde and were adsorbed on cation exchange resin, Dowex 50. They were eluted with 2 N HCl and water, dried under vacuum, and dissolved in 10% isopropanol.

The amino acids were chromatographed using methyl ethyl ketone : pyridine: water system (7 : 1.5 : 1.5), according to Kaunff et al (1959). The chromatograms were developed three times with the same solvent for 12 hrs in each run. The chromatograms were sprayed with ninhydrin and copper nitrate solution as described by Bode et al (1952). The coloured spots were cut, eluted with 5 ml methanol and their optical densities were read at 515 mu wavelength. The concentration of the amino acids were determined from standard curves of the amino acids prepared and treated as the amino acid mixture.

## RESULTS AND DISCUSSION

Satisfactory results were obtained for the determination of N-terminal amino acids of casein by labelling the free amino groups with dinitrofloro-benzene (DNFB) to give the dinitrophenyl derivatives of casein ; DNP-casein. On hydrolysis, DNP - amino acids were liberated. Paper electrophoresis was favoured (Biserte et al 1958, Lock hart et al (1954) for the determination of the water soluble DNP-amino acids, since it presented a rapid and more accurate method. Low current potential of 350 V gave good results because both DNP-arginine and E-DNP lysine separated well after 5 hrs in borate buffer, pH 9.2, as shown in Fig. 2.

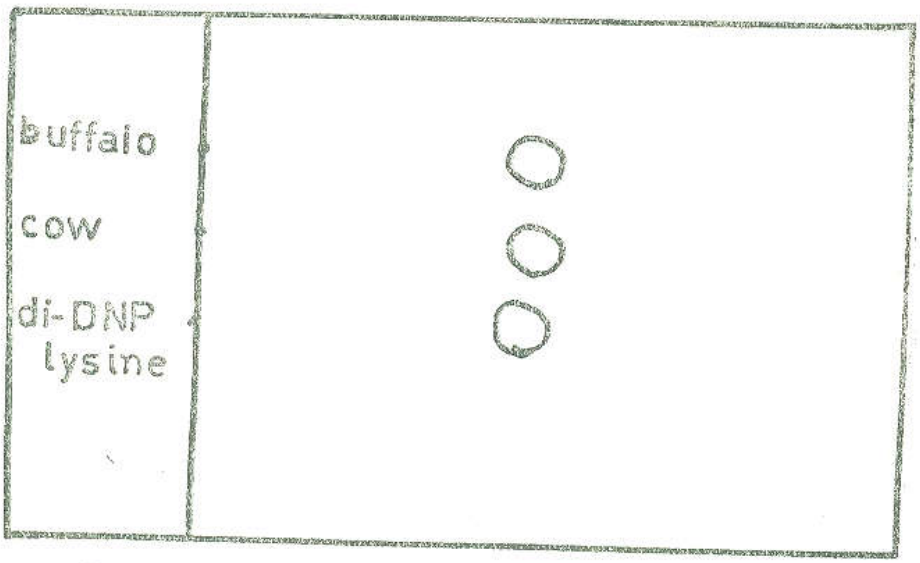


FIG. 1.—Paper chromatography of ether soluble DNP- amino acids: buffalo and cow milk casein.

Fig. (1) showed that the chromatogram of ether soluble DNP-amino acids of buffalo and cow milk caseins contained residues which were indentical to di-DNP lysine.

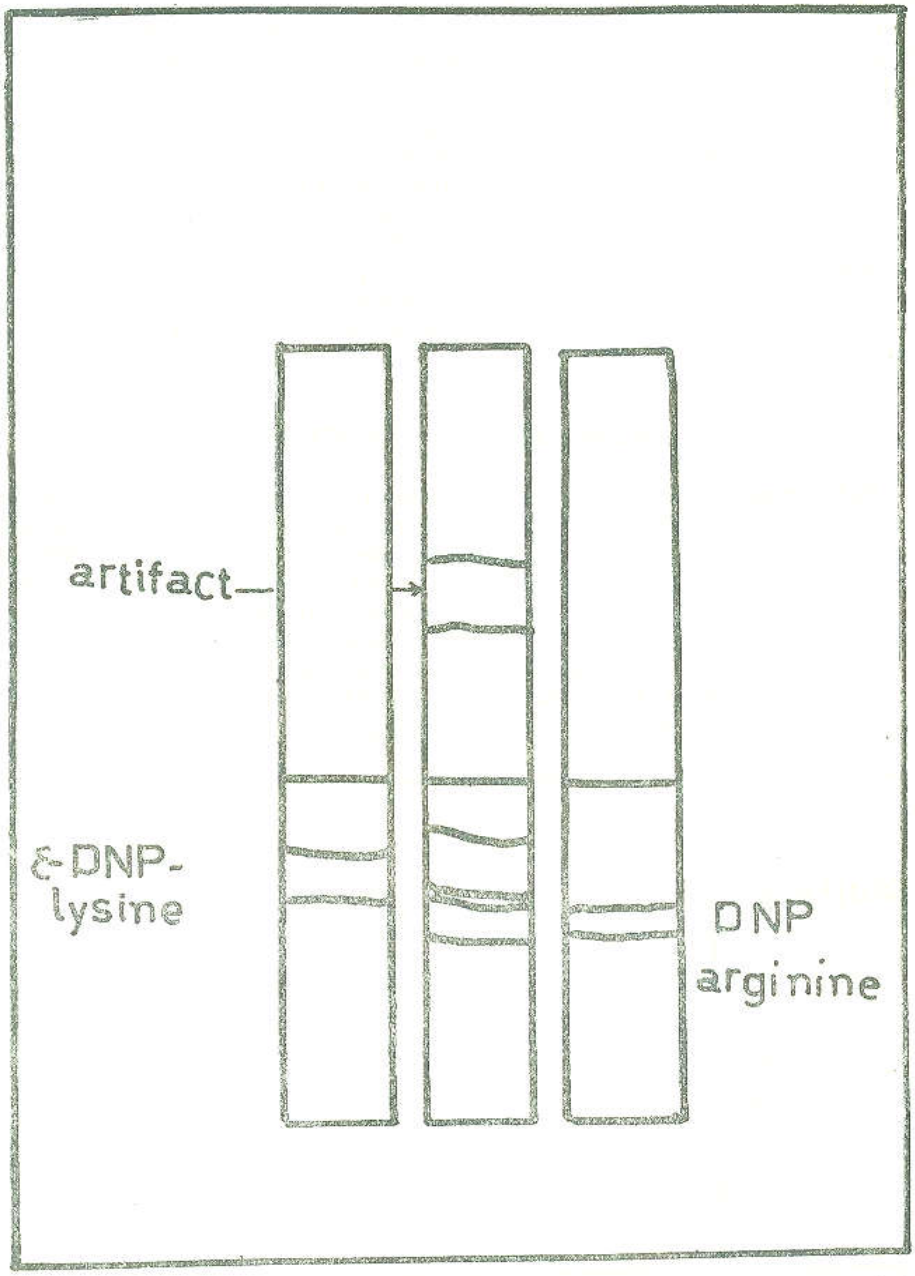


Fig. 2.—Paper eleletrophoresis of water soluble DNP-amino acids in buffalo and cow milk casein.

Fig. 2 indicated that water soluble DNP-amino acids in both caseins were indentical. There were two zones in the electropherogram. The nearer to the starting line was identical to Σ-DNP lysine, while the furth er was identical to the DNP-arginine. A third zone was found on the opposite direction which was not in accordance with any other water slouble DNP-amino-acids and accordingly considered an artifact. Therefore both caseins contained lysine and arginine residues in their N-terminal amino acids, which, were in accordance with the results of Mellon et al (1953), and Ise (1958), on the a- and B-caseins of cows. Lea and Hannan (1950), and Schwartz and Lea (1932), however, reported the presence of slight amounts of aspartyl, glutamyl, lysyl, phenylalanyl and valyl residues. The discrepancy could be explained on the basis of the difference between the samples used. These investigators used commercially prepared casein which usually contains small amounts of carbohydrate residues which would react with the protein during the elevated temperature of the drying process. Accordingly, the amount of free terminal amino acids would be reduced and gave lower values. The presence of residues other than lysine and arginine were due to traces of other proteins beside casein and to the partially splitted casein molecules present in commercial casein. In this study, casein was prepared from fresh milk and under laboratory controlled conditions to eliminate any other foreign residues.

The measure of the total absorption in alkaline solution of the unhydrolysed derivative was used in this study to determine the amount of free amino groups. Buffalo milk casein was found to contain much higher free amino groups than cows as shown in table 1, being 85 and 76 mole per $10^5$g casein respectively. Chain lysine in buffalo milk casein, however, was insignificantly higher ; average 24.45 mole, than cow milk casein ; average 22.35 mole per $10^5$ g casein.

The N-terminal lysine of buffalo casein was found to be significantly more than that of cows ; average 2.06 mole and 1.59 mole respectively. Values. in local cows were in accordance to those of Mellon et al (1953), but less than that reported by Ise (1958). The former found 1.3 and 2.1 mole in a-and B-casein respectively, while the latter reported 7.1 mole of a-casein.

There was insignificant difference between average of N-terminal arginine in both caseins. Buffalo casein contained 12.51 mole, while cows 12.09 mole, which were more than those reported by Mellon et al (1953) and Ise (1958). There was no significant difference between the average contents of N terminal amino acids in both caseins, since they were 14.57 and 13.67 mole per $10^5$ g in buffaloe's and cows; respectively.

The distribution of N-terminal amino acids differed in both caseins. For every lysyl group there were approximately 6 arginyl groups for buffaloes' and 8 for cows. Mellon et al (1953), found that in a-casein for every lysyl residue there were 7 arginyl, while in B-casein for every two arginyl residues there was one lysyl.

TABLE 1.—**Total amino groups, chain lysine, terminal lysine, terminal arginine, and total N-terminal groups in buffalo and cow milk casein.** *

| Constituent | Buffalo | | Cow | | Significance of difference |
|---|---|---|---|---|---|
| | Range | Average | Range | Average | |
| Total amino groups. | 92—79 | 85 | 83—70 | 76 | + |
| Chain lysine. . . . | 28.33—19.25 | 24.45 | 26.08—19.85 | 22.35 | — |
| Terminal lysine . . | 2.76— 1.73 | 2.06 | 1.88— 1.46 | 1.59 | + |
| Terminal arginine . | 14.52—10.08 | 12.51 | 14.42—10.16 | 12.09 | — |
| Total N-terminal groups . . . . . | 16.52—12.15 | 14.57 | 15.89—11.94 | 13.67 | — |

* All values are expressed in mole/$10^5$ g casein.

+ (significant) — (insignificant).

In this study the hydrazinolysis of caseins with some modifications was used for the identification of C-terminal amino acids instead of the enzyme hydrolysis by carboxypeptidase. The accuracy of the later method was found Fox (1945) to depend mostly on the purity of the enzyme and the different liberation rates of the amino acids. When the former method was used, clear tailing on the chromatogram was observed which was due mainly to the presence of anhydrous hydrazine. When benzaldehyde, was used to eliminate the excess of the hydrazine, it was found to be present in the aqueous solution which upset the chromatographic separation. Therefore, the amino acids were adsorbed on cation exchange resin, the benzaldehyde was washed away with water and the amino acids were eluted.

Fig. 3, and 4 showed that the C-terminal amino acids of both caseins were identical. The first spot in fig. 4 had the relative movement of aspartic acid, the second spot corresponded to glutamic acid, while the third, fourth, and fifth were identical to glycine, serine, Fig. 3, and alanine, fig. 4, respectively.

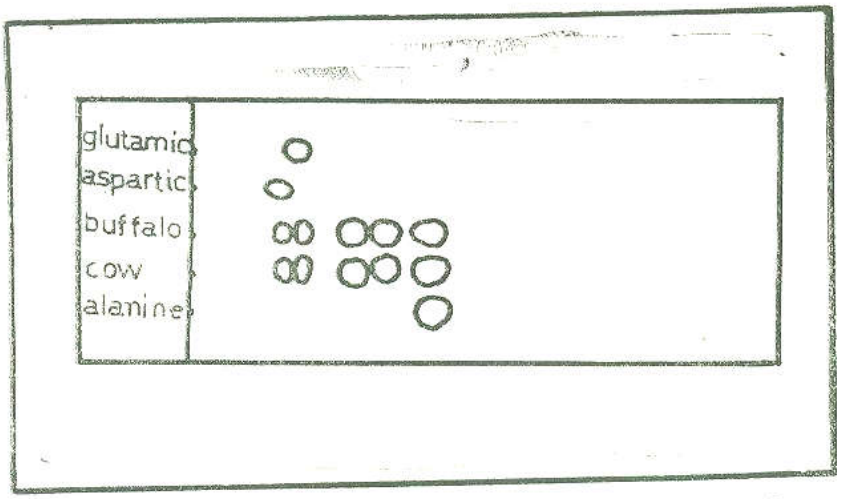


FIG. 3.—Identification of C-terminal amino acids by peper chromatography in buffalo and cow milk caseins

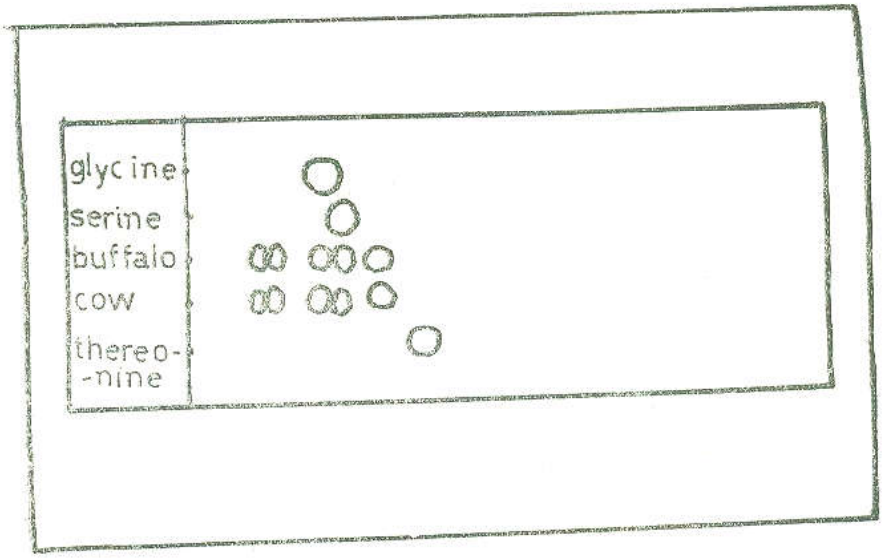


FIG. 4.—Identification of C-terminal amino acids by paper chromatography in buffalo and cow milk caseins.

The relative distribution of C-terminal amino acids in buffalo milk casein was 2.18 mole glycine, 1.25 mole alanine, 3.10 mole serine, 2.24 mole glutamic, and 3.94 mole aspartic acid per $10^5$g casein. The corresponding values for cow milk cascin were 2.20, 2.88, 3.04, 2.46 and 3.58 mole respectively, as shown in table 2.

TABLE 2.—**The relative distribution of C-terminal amino acids in buffalo and cow milk casein.** *

| Amino acid | Buffalo | | Cow | | Significance of difference |
|---|---|---|---|---|---|
| | Range | Average | Range | Average | |
| Glutamic . . . . . | 2.80— 1.77 | 2.24 | 3.00— 1.90 | 2.46 | — |
| Aspartic . . . . . | 4.54— 3.10 | 3.94 | 4.14—3.16 | 3.58 | + |
| Glycine . . . . . | 2.86— 1.74 | 2.18 | 2.66— 1.81 | 2.20 | — |
| Serine . . . . . | 3.85— 2.46 | 3.10 | 3.37— 2.17 | 3.04 | — |
| Alanine . . . . . | 3.16— 2.00 | 2.65 | 3.40— 2.00 | 2.88 | — |
| Total C-terminal amino acids . . | 16.49—11.09 | 14.16 | 15.69—11.43 | 14.16 | — |

* All values are expressed in mole/$10^5$ g casein.

— (insignificant) + (significant).

Only aspartic acid was significantly higher in buffalo casein than cows, while the differences between the rest of the amino acids were insignificant. The average of the total C-terminal amino acids were similar in both caseins, being 14.16 mole.

The results, however, showed that both N- and C-terminal residues in both caseins were the same. Statistical analysis showed that the difference between N- and C-terminal residues were insignificant in both caseins.

## REFERENCES

1. **Akabori, S., Ohno, K. and Natita, K.** (1952).—On the hydrazinolysis of proteins and peptides. A method for characterization of carboxyl-terminal amino acids in proteins. Bull. Chem. Soc. Japan, 25, 214.

2. **Biserte, G., Holleman, J., Holleman-Dehove, J., and Snutiere, P.** (1958).— "Chromatographic reviews, Progress in chromatography, electrophoresis and related methods". Vol. 2, Elesevir Pub. Co., pp 59-104.

3. **Bode, F., Hubener, H.J., Bruckner, H. and Hoeres, K.** (1952).— Determination of amino acids in paper chromatograms. Naturwissenchaften, 39, 524. Cited in C.A. (1953). 47, 9216.

4. **Fox, S.W.** (1945).—Trminal amino acids in peeptides and proteins. Adv. Protein. Chem., 2, 156-78. Acadimic Press.

5. **Ise, C.M.** (1958).—The N-terminal amino acids of a-casein and the composition of radiation sterilized casein. Diss. Abstr 19, 431. Cited in C.A., 28, 3750 (1934).

6. **Kuanff Von. H.G., Riederi, P., and Zickgref, H.** (1959).—Quantitative bestimmung von 21 freien plasma amino siuren bie gesden ver suchspnsonen. Hoppe Seyler's Z. 316, 186.

7. **Lea, C.H., and Hannan, R.S.** (1950).—Studies of the reaction between proteins and reducing sugars in the dry state II Further observation on the formation of the casein glucosee complex. Biochem. Biophys. Acta., 4, 518.

8. **Levy, A.L.** (1954).—A paper chromatographic method for the quantitative estimation of amino acids., Nature, 174, 126.

9. **Lokhart, I.M. and Abraham, E.P.** (1954).—The amino acid sequance in bactirecin A. Biochem. J., 58, 633.

10. **Mellon, E.F., Conn, A,H., and Hoover, S.R.** (1953).—The terminal amino groups of a- and B-casein. J. Am. Chem. Soc., 75, 1675.

11. **Sanger, P.E.** (1945).—The free amino groups of insulin. Biochem. J., 39, 507.

12. **Schwartz, H.M. and Lea. C.H.** (1952).—The reaction between proteins and reducing sugars in the dry state. Relative reactivity of the a-, and E-amino groups of insulin. Biochem. J., 50. 713.

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# دراسة على الأحماض الأمينية الطرفية فى كازين اللبن البقرى والجاموسى

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## الملـــخص

قدرت المجاميع الأمينية والكربوكسيلية الطرفية فى كازين اللبن البقرى والجاموسى بواســـطة الفصـــل الكـــروماتوجرافى الورقى وتميز المجمـــوعات الأمينيـــة الطرفيـــة بواســـطة اتحادهـــا بثنائى نيتروفلوروبنزين ثم فصـــلها وتم تحليل وتقدير الأحماض الكربوكسيلية الطرفية بواسطة الهيدارازين .

وتدل النتـــائج على أن كلا نوعى الكازين الجاموسى والبقرى تحتوى على المتوسطات التالية من الأحماض الطرفية ذات المجاميع الأمينية المنفردة على التوالى معبرا عنهـــا جزىء لكل ١٠ جم من الكازين ١٢ر٥١ ، ١٢ر٠٩ أرجنين ٢ر٠٦ ، ١ر٥٩ ليسين ١٤ر٥٧ ، ١٣ر٦٧ أحماض أمينية طرفية ٨٥ ، ٧٦ مجموعات أمينية منفردة كلية ، ٢٤ر٤٥ ، ٢٢ر٣٥ ليسن غير طرفى .

كذلك وجـــد ان كلا نوعى الكازين يحتوى على الأحماض ذات المجاميع الكربوكسيلية المنفردة التالية معبرا عنها جزىء لكل ١٠ر٥ جم من الكازين ٢٤ ، ٢ر٤٦ حمض جلوتاميك ٣ر٩٤ ، ٣ر٥٨ حمض اسبارتيك ٢ر١٨ ، ٢ر٢٠ جيليسين ٣ر١٠ ، ٣ر٠٤ سبرين ٢ر٦٥ ، ٢ر٨٨ الأنين .

وقد وجد أن كلا نوعى الكازين يحتوى على نفس المتوسط من الأحماض الكربوكسيلية الطرفية الكلية ١٦ ١٤ جزىء لكل ١٠ر٥ جم كازين .