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The Isolation of Phosphothreonine from Bovine Casein

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Lipmann[1] showed that at least part of the phosphoric acid present in casein is bound by an ester linkage to serine. This made it also conceivable that a part of the phosphoric acid might be coupled to threonine, although the phosphoric acid ester of this β-hydroxy acid had never been isolated from a protein hydrolysate. Moreover, since Plimmer[2] showed that synthetic phosphothreonine has about the same stability toward acid hydrolysis as phosphoserine, it seemed that it might be possible to isolate phosphothreonine from partial, acid hydrolysates of casein. A preliminary report describing efforts to isolate phosphothreonine and phosphopeptides from partial hydrolysates of casein has been given elsewhere[3]. The present paper describes the isolation of phosphothreonine.

EXPERIMENTAL

Preparation. The procedure used by Lipmann for the isolation of phosphoserine has been partially followed here. Commercial bovine casein was hydrolysed with 2 *N* hydrochloric acid for 20 h at 100° C, according to Lipmann. The hydrochloric acid was neutralized with barium carbonate and conc. ammonia, and the resulting barium complex was then precipitated by adding an equal volume of 96 % ethanol. The precipitate was collected, dried with ethanol and ether, and was thus obtained as a greyish-brown powder.

200 g of this powder was suspended in 500 ml of distilled water, and most of it was dissolved by adding 340 ml of 4 *N* hydrochloric acid. The solution was filtered and subsequently run through a 32 × 8 cm column of a sulphonated polystyrene resin (Dowex 50) of technical grade. By this procedure, most of the barium was removed. To get suitable particles, the ion exchange resin was milled in a ball mill. The larger particles were removed by passing the resin through a sieve (9 mesh per cm), and the smaller particles were separated off by sedimentation in water. The resin was used in its acid form. The rate of flow of solution through the column was about 100 ml per hour. 3 l of distilled water were then used to wash the column. The resin was regenerated with 4 *N* hydrochloric acid.

The combined effluent volumes from three experiments (corresponding to 600 g of the greyish-brown powder) were concentrated to 2 l. The solution was heated twice with charcoal to decolorize it and finally concentrated to a small volume. At this stage a crystalline precipitate appeared which was shown to consist mostly of barium phosphate. This precipitate was filtered off, and the solution was once more run through the Dowex 50 column to remove the last traces of barium.

After evaporation to a concentration of 10.9 mg total nitrogen/ml the solution was analysed using two-dimensional paper chromatography (phenol-cupron-NH_3/pyridine-isoamyl alcohol)[4]. The resulting chromatogram showed a large spot corresponding to phosphoserine and fainter spots corresponding to glutamic acid, aspartic acid, leucine, tyrosine and serine. 100 ml of the solution corresponded to about 500 g casein.

To remove the free amino acids and free phosphoric acid present, displacement chromatography according to Partridge and Brimley[5] was used. The resin Dowex 2 was milled similary to Dowex 50 and used as the free base. A column consisting of three parts was set up. The dimensions of the three resin beds were 24 × 3.5, 12 × 2.2, and 7.5 × 1.3 cm. The resin was regenerated according to Partridge and Brimley.

100 ml of the above mentioned solution containing 10.9 mg total nitrogen/ml was allowed to run slowly into the bed. The solution was subsequently displaced with 0.2 *N* acetic acid and finally 0.2 *N* hydrochloric acid. By this method it was possible to obtain three separate fractions, as checked by paper chromatography. The first fraction was displaced with acetic acid and contained the ordinary amino acids. The second fraction was eluted with acetic acid and contained acid peptides. After about 1 400 ml had run through the column, the solvent was changed to hydrochloric acid, which displaced the third fraction consisting mainly of phosphoserine. Following this fraction, free phosphoric acid was eluted from the column.

After some of the phosphoserine had been crystallized out of solution the third fraction, was concentrated to about 20 ml and applied to another column for the final separation. This column (115 × 3.6 cm) was packed with Dowex 50 (analytical grade, 250—500 mesh, 8 % crosslinking) and fitted with a waterjacket to keep the temperature at 37° C. A separation was obtained by elution with 0.01 *N* hydrochloric acid. The effluent fluid was separated into 10 ml fractions, and each fraction was tested for ninhydrin-reacting substances. By this method it was possible to obtain seven different fractions, some of which could be easily crystallized. The fourth fraction to emerge from the column, after 1 500 ml, was phosphoserine. The fifth fraction to emerge was a crystalline substance, which yielded threonine on hydrolysis, suggesting that the original substance might be phosphothreonine. The yield was about 20 mg. A study of the remaining fractions will soon be published.

Synthesis of DL-*phosphothreonine.* The barium salt of DL-phosphothreonine was synthesised according to Plimmer[2]. Barium was removed by electrodialysis and the substance further purified by running through a small Dowex 50 column (30 × 0.9 cm) at room temperature, otherwise the procedure described above was used. DL-phosphothreonine crystallized out of a water-methanol mixture in lustrous, stout prisms. The yield was low, about 5 % of the theoretical. M. p. 194° (decomp.), (Found: C 24.2; H 5.16; N 6.89; P 13.4; Calc. for $C_4H_{10}O_6NP$ (199.1): C 24.1; H 5.06; N 7.04; P 15.6).

Phosphorus was determined according to Teorell[6] with a combustion time of 4 days. As in the case of phosphoserine[7] it seems to be difficult to completely split off all the phosphorus. Further hydrolysis did not give higher values. As expected, the substance was optically inactive.

Fig. 1. Photographs (negative) of paper chromatograms of a hydrolysate of phosphothreonin (right) compared with threonine (left). Left chromatogram run with pyridine + isoamyl alcohol, the right one with phenol + cupron. The faint spot corresponds to unhydrolysed phosphothreonin.

Identification of L*-phosphothreonine.* One fraction from the Dowex 50 column gave after hydrolysis (20 h, 120° C, in sealed tubes) a ninhydrin-reacting substance with the same R_F-values as threonine. It was tested with one dimensional paper chromatography, run both with phenol + cupron and pyridine + isoamyl alcohol [4] (Fig. 1). On a one-dimensional chromatogram the fraction before hydrolysis gave a spot in the same position as synthetic phosphothreonine. In this case the chromatogram was developed with phenol + cupron.

The X-ray powder diffraction patterns from synthetic phosphothreonine and the isolated fraction were identical (Fig. 2). M. p. 194° (decomp.), (Found: C 24.4; H 5.19; N 7.03; P 14.0; Calc. for $C_4H_{10}O_6NP$ (199.1): C 24.1; H 5.06; N 7.04; P 15.6). The phosphorus value is low also in this case. Optical rotation: α_D^{24} $-0.19°$ (water; l,1; c, 2.58); $[\alpha]_D^{24}$ $-7.37°$.

X-ray crystallographic investigation. The synthetic DL-phosphothreonine and the prepared L-compound were studied with single crystal methods. Rotation-crystal diagrams and Weissenberg photographs from rotation about the c axis gave identical pictures for the two compounds. The crystals belonged to the orthorhombic system with the cell dimensions: $a = 7.74$ Å; $b = 9.28$ Å; $c = 10.74$ Å. The density was 1.72 (flotation method). A formula weight of 199.1 gives four molecules per cell.

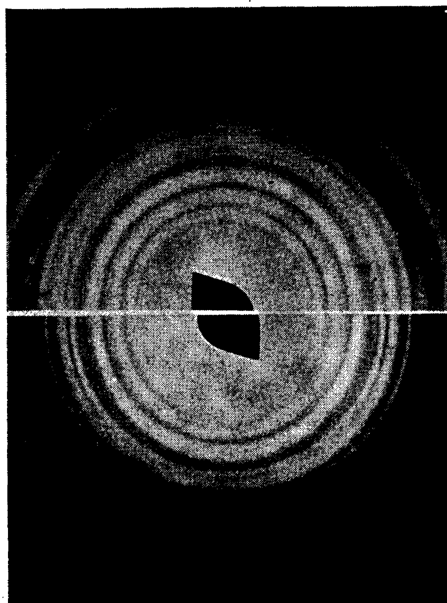

Fig. 2. X-ray diffraction patterns of synthetic DL-*phosphothreonine (above) and the isolated* L-*phosphothreonine (below).*

DISCUSSION

From the results it seems to be proved that the phosphorus in casein is not only bound to serine but also to threonine. Casein has about the same content of serine and threonine (6.3 % and 4.9 %, respectively [8]) but it is possible to isolate much greater amounts of phosphoserine than phosphothreonine. Two explanations of this fact seem possible, either a difference in the phosphorylation affinity or a difference in the stability toward acid hydrolysis. Which is the case is still a problem to be solved.

The optical rotation shows about the same values as phosphoserine, but with different sign. Phosphothreonine: $[\alpha]_D^{24}$ $-7.4°$; $[M]_D^{24}$ $-14.6°$. Phosphoserine: $[\alpha]_D^{23}$ $+ 7.2°$ *; $[M]_D^{23}$ $+13.5°$. The free amino acids threonine and serine also rotate the polarized light in opposite directions.

The identical X-ray diagrams of the optically active and the racemic form of phosphothreonine might indicate that the DL-form is a conglomerate. This is the case for the unphosphorylated amino acid [9].

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* The value earlier reported [7], referred to the specific rotation. Owing to an error in proofreading, the parentheses around the letter *a* are missing.

SUMMARY

L-phosphothreonine has been prepared from an acid hydrolysate of bovine casein. It has been characterized by its position on paper chromatograms, by elementary analysis, melting point, optical rotation and the dimensions of the unit cell.

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