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TRANSFORMATION OF DEHYDROALANINE RESIDUES IN CASEIN TO S-β-(2-PYRIDYLETHYL)-L-CYSTEINE SIDE CHAINS

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The SH group of 2-mercaptoethylpyridine adds to the double bond of dehydroalanine residues in alkali-treated casein and acetylated casein to form S-β-(2-pyridylethyl)-L-cysteine (2-PEC) side chains. The generated cysteine derivative can be assayed, after protein hydrolysis, by standard amino acid analysis techniques. The novel transformation may be useful for assessing chemical and biological functions of dehydroalanine residues in proteins.

INTRODUCTION

Exposing proteins to alkali induces elimination reactions of serine and cystine to dehydroalanine side chains (1-3). The double bond in dehydroalanine is known to combine with ε-NH_2 groups of lysine to form lysinoalanine and with the SH group of cysteine to form lanthionine residues, respectively (1-3), as illustrated in Figure 1. Dehydroalanine residues also occur naturally in peptide antibiotics such as subtilin (3). In this paper we report the synthesis of 2-mercaptoethyl-pyridine and its reaction with the double bond of dehydroalanine residues in casein and acetylated casein.

MATERIALS AND METHODS

S-β-(2-Pyridylethyl) thiolacetate (2-PETA, Figure 1). To 21 g (0.2 moles) of redistilled 2-vinylpyridine (Aldrich) in 75 cc methanol was added dropwise with stirring at room temperature a solution of 15.2 g (0.2 moles) of thiolacetic acid (Eastman) in 75 ml methanol. The mixture was left standing overnight, briefly refluxed for about five minutes, and the solvent removed on a rotatory evaporator. The residual oil was purified by vacuum fractional distillation. The main fraction (32.6g) distilling at 80°C and 0.2 mm Hg was collected. The structure of the product was confirmed by nuclear magnetic resonance spectroscopy (NMR). Specific gravity = 1.122 at 20°C.

S-β-(2-Pyridylethyl) mercaptan (2-PEM, Figure 1). To 27.2 g (0.15 moles) of 2-PETA was added a solution of 12.6 g (0.32 moles) NaOH in 100 ml of H_2O. The reaction mixture was stirred for 1 hr, decolorized with charcoal, filtered, and the clear filtrate neutralized with 25 ml conc. HCl. The separated oil was collected. An additional amount of the oil was extracted from the aqueous layer with dichloromethane. The combined oil fractions were dissolved in dichloromethane. The solution was washed three times with

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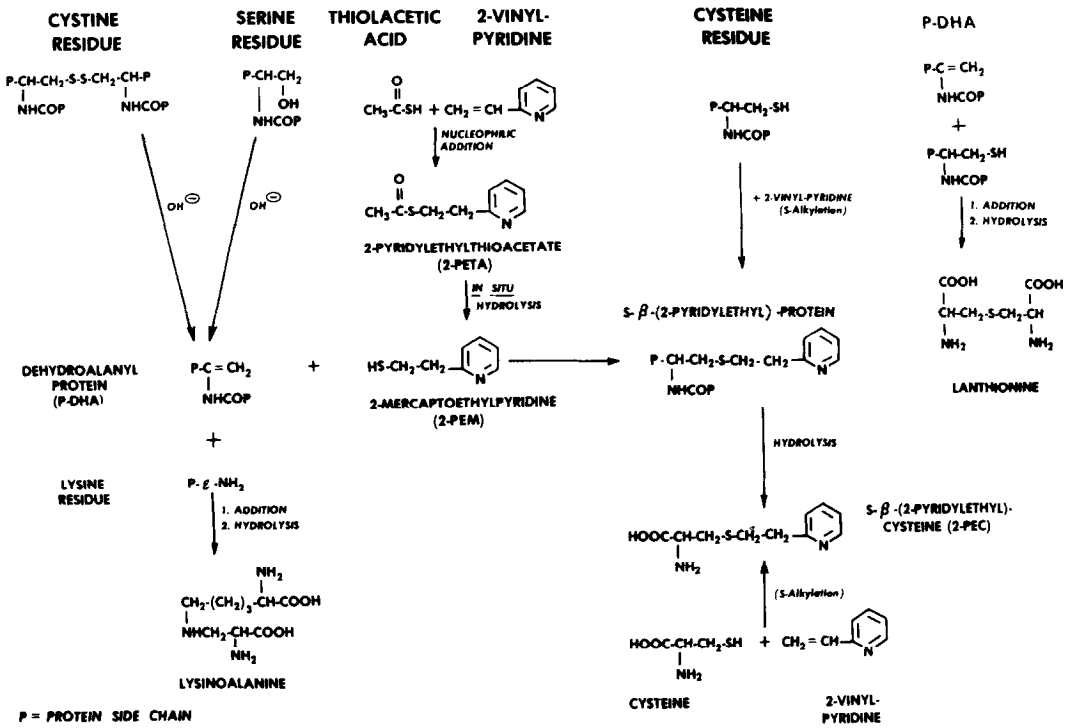

Figure 1. Transformation of dehydroalanyl to lysinoalanine (LAL), S-β-(2-pyridylethyl)-L-cysteine (2-PEC), and lanthionine (LAN) residues in a protein. Hydroxide ions induce elimination reactions in cystine and serine to produce dehydroalanine. The double bond of dehydroalanine can then interact with the ε-NH_2 group of lysine to form LAL, with the SH group of cysteine to give LAN, and with the SH group of added 2-mercaptoethylpyridine to form 2-PEC, as illustrated. The latter is identical with the compound obtained from cysteine and 2-vinlypyridine.

small volumes of water and dried over Na_2SO_4. After evaporation of the dichloromethane, the residue was purified by fractional vacuum distillation. The fraction (14.1 g) distilling at 48°C and a pressure of 0.18 mm Hg was collected. The structure was confirmed by NMR. Specific gravity = 1.082 at 20°C.

Acetylated Casein. The procedure was adapted from Riordan and Vallee (4). To 5 g of commercial casein (American Casein Corporation) was added 100 ml distilled H_2O and 100 ml saturated sodium acetate solution at 0°C. The suspension was cooled in an ice-bath and 10 ml of acetic anhydride were added dropwise with stirring over 2 hours. The mixture was stirred another hour, dialyzed against 0.01 N acetic acid, and lyophilized.

Protein Reactions. These were carried out under a nitrogen atmosphere with 1% (w/v) solutions of casein or acetylated casein in 0.1 N NaOH for 3 hrs at 70°C. The thiol reagent was added at a 0.5 mg/mg protein ratio; sufficient NaOH was added after thiol addition to maintain the pH between 12 and 12.5. With 2-PETA, the pH was monitored during the treatment and alkali added to maintain pH as de-esterification progressed (simultaneous treatment). At the end of treatments, sample solutions were cooled to 20°C and adjusted with acetic acid to pH 9.0, then dialyzed and lyophilized.

The following conditions were used in a sequential addition of the thiol after alkali-treatment. The protein was first exposed to alkali, as

described above. The isolated, alkali-treated protein was dissolved in pH 7.8, 0.2 molar Tris-HCl buffer at 20°C. Thiol was added and the reaction mixture left standing under nitrogen for 16 hours, dialyzed, and lyophilized.

Amino Acid Analyses. These were carried out on a single-column Durrum Model D-500 analyzer with a reproducibility of ± 2% or better as described previously (5).

RESULTS AND DISCUSSION

An ideal reagent for modifying dehydroalanine residues in proteins should meet the following requirements: (a) It should selectively and rapidly modify the double bond of dehydroalanine; (b) The modified derivative should survive acid hydrolysis conditions used for proteins; (c) The derivative should be eluted in a convenient position as a well-resolved peak in standard amino acid analysis. Since previous studies (6) have shown that S-β-(2-pyridylethyl)-L-cysteine (2-PEC), derived from alkylating of half-cystine residues by 2-vinylpyridine, appears to meet conditions (b) and (c), it remained to be shown that dehydroalanine residues can be transformed to 2-PEC side chains.

In principle, 2-mercaptoethylpyridine (2-PEM) can add to the double bond of dehydroalanine to form 2-PEC side chains. (This reaction can be viewed as complementary to the formation of 2-PEC from cysteine and 2-vinylpyridine). 2-PEC would also be produced from 2-PETA and dehydroalanine, if the S-acetyl groups were hydrolytically cleaved *in situ* during the reaction (Figure 1). We chose casein as a model protein to evaluate these transformations because it does not have any disulfide bonds that may interact with 2-PEM. In addition, we included acetylated casein in this study because it has already been shown that acetylation before alkali treatment prevents lysinoalanine formation but not racemization following alkali-treatment (7). Exposing of acetylated casein to alkali, should, therefore, generate dehydroalanine residues that cannot react with the protected ε-NH_2 groups to form lysinoalanine. Added 2-PEM should thus trap the dehydroalanine side chains, as outlined in Figure 1.

Results in Tables 1 and 2 show that treating casein or acetylated casein with 0.1 N NaOH at 70°C for 3 hours in the presence of either

Table 1. Simultaneous and sequential treatment of casein with alkali and 2-pyridylethylmercaptan (2-PEM)[a,b]

Amino acid	Untreated casein	Casein in buffer + 2-PEM	Casein + alkali	Casein + alkali + 2-PEM (simultaneous)	Casein + alkali + 2-PEM (sequential)
THR	5.02	5.01	2.62	4.46	2.70
SER	6.80	6.73	2.94	4.79	3.05
ILEU	6.21	6.30	6.10	6.09	6.05
LEU	11.61	11.74	12.05	11.69	11.91
HIS	3.58	3.63	3.22	3.62	3.40
LYS	9.60	9.64	5.63	9.37	5.59
ARG	4.48	4.45	3.81	4.00	3.74
LAL	0.00	0.00	8.37	0.78	9.11
2-PEC	0.00	0.00	0.00	4.39	0.33

a/ Conditions: 1% (w/v) protein in 0.1 N NaOH under nitrogen; 70°C; 3 hrs. 2-PEM was added simultaneously or sequentially as described in the experimental section.

b/ Numbers are grams per 16 g nitrogen, i. e. approximately g per 100g protein.

2-pyridylethylmercaptan or its S-acetyl derivative prevents lysinoalanine formation compared to analogous treatment without the mercaptan. The results also show that the decrease in lysinoalanine formation was accompanied by the appearance of a new amino acid whose elution position on a chromatogram of an amino acid analyzer (4) is identical to that of an authentic sample of S-β-2(pyridylethyl)-L-cysteine (2-PEC), derived from reaction of cysteine with 2-vinylpyridine. These observations support a reaction scheme shown in Figure 1.

Additional studies revealed that simultaneous treatment of acetylated casein with 2-PEM produced 4.31% 2-PEC compared to a 1.39% yield observed after sequential treatment, where 2-PEM is added to acetylated casein after alkali-treatment. These results imply that 2-PEM prevents hydrolytic destruction of some of the dehydroalanine residues during the alkali treatment, trapping them as 2-PEC.

Since alkali-treatment of acetylated casein prevents lysinoalanine formation but not amino acid racemization (6), and since the results of the

Table 2. Simultaneous and sequential treatment of acetylated casein with alkali and S-acetylpyridylethylmercaptan, 2-PETA.[a,b]

Amino acid	Acetylated casein (untreated control)	Acetylated casein + alkali	Acetylated casein + alkali + 2-PETA (simultaneous)	Acetylated casein + alkali + 2-PETA (sequential)
THR	3.52	2.87	3.36	2.76
SER	4.83	2.82	3.39	2.64
ILEU	4.28	4.23	4.31	4.08
LEU	7.87	8.48	8.40	8.17
HIS	2.75	2.27	2.46	2.46
LYS	7.02	7.09	6.99	6.91
ARG	3.05	2.64	3.11	2.62
LAL	0.00	0.84	0.00	0.94
2-PEC	0.00	0.00	4.31	1.39

a/ Conditions: 1% (w/v) protein in 0.1 N NaOH 3 hrs; 70°C.

b/ Numbers are grams per 16 grams nitrogen, approximately g/100g protein.

present study show that alkali-treatment of acetylated casein also generates dehydroalanine residues, discrimination of individual chemical, nutritional, and toxicological effects of the unnatural lysinoalanine, D-amino acid, and dehydroalanine residues in casein and other proteins appears possible. This aspect is under study.

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