A One-Step Process for Incorporation of L-Methionine into Soy Protein by Treatment with Papain

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The plastein reaction is known to be applicable to covalently incorporating essential amino acids into proteins. However, this reaction requires two enzymatic processes: protein degradation and resynthesis. In the present study we have simplified the process and made it possible to incorporate L-methionine ethyl ester (L-Met-OEt) into soy protein isolate (SPI) by its one-step treatment with papain (EC 3.4.4.10). A most preferable set of the conditions for the one-step process was established as follows: concentration of SPI (protein content: 92.0%) in a reaction medium, 20 wt %; initial pH of the medium, 10.0; ratio of papain [3.24 BAPA units mg^{-1} min^{-1} (25 °C, pH 6.2)] to SPI, 1:100 (w/w); concentration of L-cysteine (activator), 1 mM; and incubation time, 8 h (at 37 °C). When a 20:1 (w/w) mixture of SPI and L-Met-OEt·HCl was incubated under this set of conditions, it was observed that 81.5 molar % of the original L-Met-OEt was utilized to be incorporated. DL-Met-OEt could be used for the incorporation of L-methionine since the D isomer remained intact during the enzymatic process.

The plastein reaction has been studied in detail, especially for its application to food protein improvement (Fujimaki et al., 1970; Arai et al., 1975; Yamashita et al., 1976a; Fujimaki et al., 1977). In particular, this reaction can have effective use in covalently incorporating amino acids (ester forms) into peptides (Yamashita et al., 1971; Yamashita et al., 1972; Arai et al., 1974; Yamashita et al., 1975; Yamashita et al., 1976b; Arai et al., 1976; Aso et al., 1977). A typical example has first appeared in the paper that reports the papain-catalyzed incorporation of methionine to enhance the sulfur-containing amino acid level of soy protein (Yamashita et al., 1971). A subsequent study has proposed an improved method for producing a methionine-incorporated plastein on an enlarged scale (Arai et al., 1974). However, when receiving such a practical application, the conventional plastein reaction may be confronted with a degree of economic limitation since the process is inevitably composed of two different steps (Scheme I). The first-step reaction is generally carried out at a very low concentration of substrate (protein) and the second at a very high concentration of substrate (hydrolysate) in the presence of methionine ester added in the reaction system (Yamashita et al., 1971). However, if a proper set of reaction conditions is used, it may be possible to unify both processes and facilitate the methionine incorporation into protein by one step (Scheme II). Though the product cannot be called plastein in a strict sense, this simplified process would be more economical than the conventional plastein reaction and more useful to prepare a product in which methionine has been covalently enriched.

The present paper describes the reaction conditions most preferable and convenient for the incorporation of L-methionine ethyl ester into soy protein isolate by its one-step treatment with papain.

The following abbreviations are used: BAPA, *N*-benzoyl-L-arginine *p*-nitroanilide; Met-OEt, methionine ethyl ester; SPI, soy protein isolate; $[S]_0$, initial concentration of substrate (SPI) in the reaction medium; $[M]_0$, initial concentration of Met-OEt·HCl; $[M]_t$, its concentration after t (minute or hour) from the initiation; $[M]_0/[S]_0$, initial ratio (w/w) of Met-OEt·HCl to substrate (SPI); $[E]_0$, initial concentration of enzyme (papain); $[E]_0/[S]_0$, initial ratio (w/w) of enzyme to substrate; $[A]_0$, initial concentration of activator (L-cysteine); pH_0, initial pH of the reaction mixture; pH_t, its pH after incubation for t (minute or hour); t, incubation time (minute or hour); and T, incubation temperature (°C).

Scheme I

protein
↓ enzymatic protein hydrolysis
hydrolysate
↓ plastein synthesis in the presence of methionine ester
methionine-incorporated plastein

Scheme II

protein
↓ enzymatic treatment in the presence of methionine ester
methionine-incorporated product

EXPERIMENTAL SECTION

Substrate. A commercially available SPI product (Fujipro R; Fuji Oil Mill Co., Osaka) was treated with a 100-fold amount of 0.1 N NaOH for 2 h at room temperature. After removal of a minor insoluble substance by centrifugation, the soluble fraction was adjusted down to pH 4.5 with HCl to obtain a precipitate. This was collected by centrifugation for 20 min at 3000 rpm and dialyzed with a cellophane tube in running water at 5 °C for 3 days. The nondiffusible fraction was freeze-dried to obtain an alkali-denatured, acid-precipitated SPI fraction with a protein content (N × 6.25) of 92.0% on a dry-matter basis. This SPI was used as the substrate throughout the study.

Methionine Ethyl Ester (Met-OEt). Reagent grade preparations of D-methionine, L-methionine, and racemic DL-methionine (Ajinomoto Co., Tokyo) were esterified with ethanol by the method of Boissonnas et al. (1956). Each reaction product was recrystallized from ethanol–ether–hexane (2:7:1) to obtain D-, L-, or DL-Met-OEt·HCl in needle, which gave a single spot with an R_f value of 0.65 when checked by thin-layer chromatography (Wollenweber, 1962).

Enzyme. A papain preparation presented by Miles Laboratories, Inc. (Indiana) was used. Its activity for BAPA was 3.24 units mg^{-1} min^{-1} (25 °C; pH 6.2) when

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0021-8561/79/1427-0052$01.00/0

assayed according to the standard method (Arnon, 1970). The papain activity was determined also for SPI solutions at various pH values by the method of Yemm and Cocking (1955). The details are described later.

Activator. A reagent grade preparation of L-cysteine (Ajinomoto Co., Tokyo) was used as the activator for papain.

Enzymatic Process. SPI (1 g) was kneaded with a small amount of an NaOH solution so as to be a homogeneous sol or gel showing a nearly expected pH value. Met-OEt·HCl was dissolved in a given amount of water. This solution was adjusted to the similar pH value with another given amount of an NaOH solution and then mixed with the SPI sol or gel. The mixture was readjusted up to an expected pH value and, at the same time, up to a fixed quantity by adding a known amount of dilute NaOH. Thereto was added an aqueous solution containing given concentrations of papain and L-cysteine. The incubation was carried out at 37 °C for a stated period. Further details are described in the legends to the figures and tables in respective cases.

Determination of Methionine Incorporation. After the incubation, each reaction mixture, which initially contained 1 g of SPI, was filled up to 100 mL with 0.1 N NaOH and allowed to stand for 5 h at room temperature. In that case it was confirmed that the NaOH treatment had completely hydrolyzed remaining Met-OEt to free methionine. An aliquot was pipetted from the alkali-treated mixture, put in a ninefold volume of 0.2 M citrate (pH 2.2) and directly let in an amino acid autoanalyzer (Hitachi KLA-5) to determine a free methionine content. The determined value was corrected by the dilution fold to obtain a total millimolar concentration ($[M]_t$) of nonpeptide methionine and intact Met-OEt remaining in the reaction mixture after the incubation. The percent incorporation of methionine can thus be calculated from

$$\{([M]_0 - [M]_t)/[M]_0\} \times 100$$

where $[M]_0$ is given on a millimolar basis. Every determination was duplicated to present a mean value.

Differential Determination of D and L Isomers of Methionine. A reaction mixture which initially contained 1 g of SPI was filled up to 20 mL with 0.1 N NaOH and after 5 h, up to 100 mL by addition of absolute ethanol. The resulting suspension was centrifuged for 30 min at 10000 rpm to separate the insoluble fraction which carried no significant amount of free methionine. An aliquot of the supernatant was neutralized to pH 7 with HCl, dried in vacuo, and dehydrated with P_2O_5. The dehydrated sample was subjected to a trimethylsilylation–butylation treatment (Parr and Howard, 1971) and analyzed for the D and L isomers by gas chromatography. Details for the equipments and conditions of operation are described in the legend to the figure.

RESULTS

Substrate Concentration. To investigate the effect of $[S]_0$ on the methionine incorporation an experiment was carried out with $[M]_0/[S]_0$ and pH_0 kept constant. The pH_0 was tentatively set at 6.0 according to the previous study on the methionine incorporation by the conventional plastein reaction (Yamashita et al., 1971). No significant change in pH was observed as far as the incubation was initiated at pH 6.0. The rate of incorporation of L-methionine under this pH condition was found to be greatly dependent on $[S]_0$, with the highest rate of 56.2% at $[S]_0 = 20$ (Table I).

Initial pH and Its Decrease. Next, the initial velocity of the L-methionine incorporation was determined as a function of pH_0. Every run of the incubation experiment was made at $[S]_0 = 5$ (wt %), because a higher $[S]_0$ caused technical difficulty in determining an exact initial velocity. A bell-shape curve showing a pH–velocity relation was obtained with a maximum point in the neighborhood of pH 9 (Figure 1). This profile was distinctly different from that observed for the initial velocity of hydrolysis of SPI ($[S]_0 = 5$) by papain at various pH (Figure 1).

Table I. Incorporation of L-Methionine at Different Substrate Concentrations ($[S]_0$) with Other Factors Kept Constant[a]

$[S]_0$, wt %	incorporation, %
1	24.4
5	30.7
10	52.1
20	56.2

[a] $[M]_0/[S]_0 = 0.05$, $[E]_0/[S]_0 = 0.01$, $[A]_0 = 10.0$ (mM), $pH_0 = 6.0$, $t = 24$ (h), $T = 37$ (°C).

Table II. Incorporation of L-Methionine as Functions of Initial and Final pH Values (pH_0 and pH_t) with Other Factors Kept Constant[a]

pH_0	pH_t	incorporation, %
11.0	7.7	35.6
10.0	5.8	81.5
9.0	4.9	80.1
8.0	4.7	79.0
7.0	4.5	73.3
6.0	4.5	56.2

[a] $[S]_0 = 20$, $[M]_0/[S]_0 = 0.05$, $[E]_0/[S]_0 = 0.01$, $[A]_0 = 10.0$ (mM), $t = 24$ (h), $T = 37$ (°C).

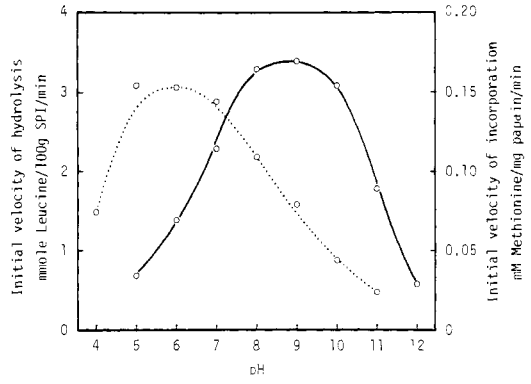

Figure 1. Initial velocities of soy protein hydrolysis (dotted curve) and of L-methionine incorporation (solid curve) by papain as a function of pH: $[S]_0 = 5$ (wt %), $[M]_0/[S]_0 = 0.05$ (w/w), $[E]_0/[S]_0 = 0.01$ (w/w), $[A]_0 = 10.0$ (mM), $t = 30$ (min), $T = 37$ (°C).

The effect of pH_0 on the methionine incorporation was investigated at $[S]_0 = 20$, since this level was found most preferable in respect of $[S]_0$ (Table I). No buffer was used because such a very high $[S]_0$ made it technically impossible to use it. Hence, a more or less degree of pH change was inevitable during the incubation and, therefore, the methionine incorporation was determined as functions of both pH_0 and pH_t. The highest incorporation was attained when pH_0 was set at 10.0 with a decrease to pH 5.8 after 24 h from the initiation (Table II).

Incubation Time. The time-course incorporation of L-methionine was investigated under the most preferable conditions: $[S]_0 = 20$ and $pH_0 = 10.0$. The percent incorporation approached to a plateau in 24 h from the initiation, with the almost same rate of incorporation at $t = 48$ h (Figure 2).

Concentration of L-Methionine Ethyl Ester. The effect of $[M]_0$ on the incorporation of L-methionine was studied at $[S]_0 = 20$, $pH_0 = 10.0$, and $t = 24$ (h). The most

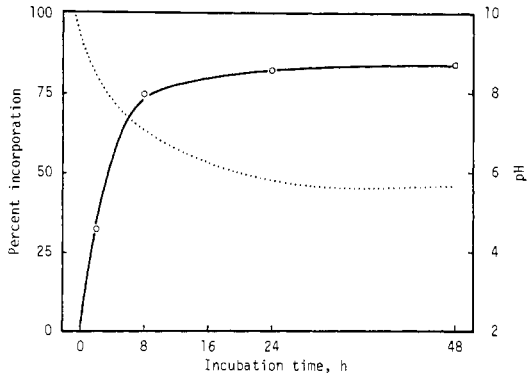

Figure 2. Time courses of pH change (dotted curve) and L-methionine incorporation (solid curve) by papain: $[S]_0$ = 20 (wt. %), $[M]_0/[S]_0$ = 0.05 (w/w), $[E]_0/[S]_0$ = 0.01 (w/w), $[A]_0$ = 10.0 (mM), pH_0 = 10.0, T = 37 (°C).

Table III. Incorporation of L-Methionine at Different Ratios of L-Methionine Ethyl Ester Hydrochloride to Substrate ($[M]_0/[S]_0$) with Other Factors Kept Constant[a]

$[M]_0/[S]_0$	pH_0	pH_t	incorporation, %
0.01	10.0	5.6	61.0
0.05	10.0	5.8	81.5[b]
0.10	10.0	6.1	80.0
0.20	10.0	6.3	75.3

[a] $[S]_0$ = 20, $[E]_0/[S]_0$ = 0.01, $[A]_0$ = 10.0 (mM), t = 24 (h), T = 37 (°C). [b] From Table II.

Table IV. Incorporation of L-Methionine at Different Ratios of Enzyme to Substrate ($[E]_0/[S]_0$) with Other Factors Kept Constant[a]

$[E]_0/[S]_0$	incorporation, %
0.001	19.9
0.005	78.0
0.01[b]	81.5[c]
0.02	85.0

[a] $[S]_0$ = 20, $[M]_0/[S]_0$ = 0.05, $[A]_0$ = 10.0 (mM), pH_0 = 10.0, t = 24 (h), T = 37 (°C). [b] The enzyme activity after the incubation for 24 h represented 65.0% of that before the incubation, when assayed by the method of Arnon (1970). [c] From Table II.

efficient incorporation was attained at $[M]_0/[S]_0$ = 0.05 (Table III).

Enzyme–Substrate Ratio. When an experiment was carried out with varying initial amounts of enzyme under the conditions fixed at $[S]_0$ = 20, $[M]_0/[S]_0$ = 0.05, pH_0 = 10.0, and t = 24 (h), the methionine incorporation was increased with increasing $[E]_0/[S]_0$. However, in spite of the twofold increase of $[E]_0/[S]_0$ from 0.01 to 0.02, the incorporation rate was not improved to such an extent (Table IV). Greatly different from this tendency, the incorporation rate observed when $[E]_0/[S]_0$ was made half from 0.01 to 0.005 represented approximately 90% of that observed at $[E]_0/[S]_0$ = 0.01. The amount of the enzyme equivalent to 1% of that of the substrate was thus found to be nearly adequate even by taking into consideration the enzyme inactivation during incubation, since the activity after the 24-h incubation still accounted for 65.0% of the initial activity (3.24 BAPA units mg^{-1} min^{-1}) as described in the footnote of Table IV.

Activator Concentration. All the above experiments were conducted at $[A]_0$ = 10.0 (mM). To find out an irreducible minimum of $[A]_0$, another experiment was made at different $[A]_0$ with other factors kept constant. It was seen that the employed papain preparation had been partially activated since the enzyme permitted a degree of methionine incorporation even without addition of L-cysteine (Table V). Its addition up to 1.0 mM resulted in a satisfactory degree of the methionine incorporation; this degree almost entirely represented that observed at $[A]_0$ = 10.0 (Table V).

Table V. Incorporation of L-Methionine at Different Concentrations of Activator ($[A]_0$) with Other Factors Kept Constant[a]

$[A]_0$, mM	incorporation, %
0	43.9
0.5	66.5
1.0	78.0
2.0	82.0
5.0	80.9
10.0	81.5[b]

[a] $[S]_0$ = 20, $[M]_0/[S]_0$ = 0.05, $[E]_0/[S]_0$ = 0.01, pH_0 = 10.0, t = 24 (h), T = 37 (°C). [b] From Table II.

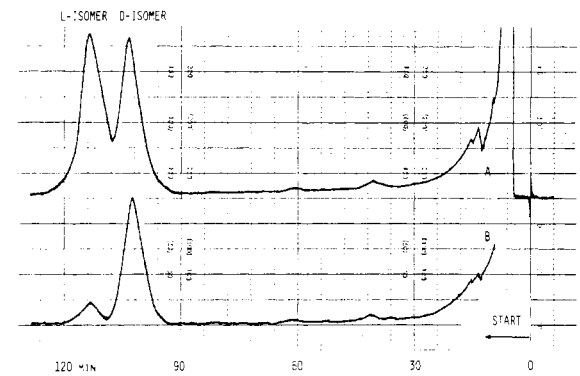

Figure 3. Resolution by gas chromatography of D and L isomers of *N*-trifluoroacetylmethionine isopropyl ester. Chromatograms A and B show resolution patterns obtained with samples before and after incubation, respectively. Apparatus: Hitachi F-6 gas chromatograph equipped with a flame ionization detector. Column: stainless steel column (0.25 mm × 10 m) coated with *N*-trifluoroacetyl-L-valyl-L-valine cyclohexyl ester. Conditions: injector temperature, 220 °C; column temperature, 130 °C; detector temperature, 230 °C; flow rate of carrier gas (N_2), 15 mL/min.

D-Methionine Remaining Unincorporated. A study was carried out using DL-Met-OEt·HCl, with all the described factors kept constant at their most preferable levels. The reaction mixtures before and after the incubation for 24 h were quantitatively analyzed for D- and L-methionine remaining unincorporated. Gas chromatography clearly resolved *N*-trifluoroacetyl-D-methionine isopropyl ester and its L isomer. The upper chromatogram in Figure 3 shows the two peaks covering almost the same areas, and the peak area of the D isomer was exactly similar to that of the D isomer from the sample after the 24 h incubation (lower chromatogram of Figure 3). This result indicates that the D-Met-OEt fraction of the racemate makes no contribution to the present process with papain. Assuming that the racemate per se is a 50:50 mixture of D- and L-Met-OEt, it is calculated that the D and L isomers, either in free or in ester form, remain in the incubated mixture with a ratio of 100:15. This datum on the remaining amount of the L-isomer is well consistent with the result that 81.5% of the original L-Met-OEt is consumed to be incorporated (Table II).

Hydrolysis of D- and L-Methionine Ethyl Ester. Two experiments were conducted: the first with D-Met-OEt·HCl and the second with L-Met-OEt·HCl. Both experiments employed the most preferable set of conditions: $[S]_0$ = 20, $[M]_0/[S]_0$ = 0.05, $[E]_0/[S]_0$ = 0.01, $[A]_0$ = 1.0, pH_0 = 10.0, and t = 24 (h). An aliquot from each reaction mixture after incubation was diluted by directly adding an appropriate amount of 0.2 M citrate (pH 2.2)

Table VI. States and Quantities of D- and L-Methionine after Incubation[a]

state	quantity, % D-methionine	quantity, % L-methionine
incorporated	0.0	81.5[b]
unincorporated		
free form	1.8	17.4
ethyl ester form	98.2	1.1

[a] $[S]_0 = 20$, $[M]_0/[S]_0 = 0.05$, $[E]_0/[S] = 0.01$, $[A]_0 = 10.0$ (mM), $t = 24$ (h), $T = 37$ (°C). [b] From Table II.

and let in the amino acid autoanalyzer to determine a free methionine content. Another aliquot from the same sample was once treated with alkali as mentioned before to break down the remaining ethyl ester linkage and then submitted to the methionine determination with the autoanalyzer. The Met-OEt content was thus obtained from these two determinations by subtraction. The percent incorporation of L-methionine was 81.5% as shown in Table II, the remainder consisting mostly of free L-methionine (Table VI). On the other hand, it was found that D-Met-OEt remained almost completely intact without undergoing hydrolysis to free D-methionine to any appreciable extent (Table VI).

DISCUSSION

Modern kinetic studies have elucidated that most protease-catalyzed reactions are generally characterized by the formation of an acyl–enzyme intermediate to liberate the first product and by the subsequent occurence of the acyl–enzyme hydrolysis with formation of the second product (Glazer and Smith, 1971). When an amino compound is present in the reaction system, it is possible that this acts as a nucleophilic replacement amine and interacts with the acyl–enzyme to form a ternary enzyme–substrate–nucleophile complex which eventually gives rise to the third product. The process involving such an action by a replacement amine is generally called aminolysis (Connorsand and Bender, 1961; Jencks, 1970).

In the present one-step process there may be a high possibility that L-Met-OEt acts as a replacement amine for an acyl-papain, more exactly for a peptidyl-papain. This kind of aminolysis reaction would lead to the formation of a peptide with a methionine residue incorporated at the C-terminal. There is another possibility that L-Met-OEt first reacts with papain to form a methionyl–enzyme intermediate which subsequently undergoes aminolysis by a peptide as a nucleophile. This reaction can produce a peptide which bears a methionine residue at the N-terminal. Although L-Met-OEt is thus considered to act either as a replacement amine or as an acylation reagent, it is speculated from a related study (Yamashita et al., 1972) that the aminolysis activity of L-Met-OEt is predominant over its acylation activity.

Interest is taken in the pH region specific to effective incorporation of L-methionine, because this region is clearly different for that required for the hydrolysis of SPI by papain (Figure 1). It has been known for a long time that papain as a sulfhydryl protease is most active at a neutral or slightly acidic pH (Reed, 1966). However, as far as the unit process of aminolysis is concerned, a more alkaline condition must be more favorable since the acyl-papain as a thioester can be attacked more easily by the unionized amino group of a replacement amine. While such an alkaline pH is preferable in a chemical sense on one hand, it may affect the enzyme stability itself on the other. A pH condition most favorable for papain in an overall point of view may thus depend on both the chemical and the enzymological preference. The result from the present study suggests that the most preferable condition for the aminolysis lies around pH 9, assuming that L-Met-OEt acts as a nucleophilic replacement amine. When the reaction system was initially adjusted to pH 10.0 for the highest rate of incorporation of L-methionine (Table II), it was observed that the pH decreased down to 7.6 within the first 8 h (Figure 2). The thereby observed rate of incorporation (75.1%) represented more than 90% of the rate of incorporation (82.0%) that was found after 48 h from the initiation. This result also supports that the most preferable condition lies in the neighborhood of pH 9.

The irreducible minimum amount of enzyme may always be a most important factor for the one-step process as well as for the classical plastein reaction, especially an economical point of view. Table IV shows that the satisfactory incorporation of L-methionine requires the papain preparation amounting to at least 1% of SPI, where the enzyme has an activity of 3.24 BAPA units mg^{-1} min^{-1} (25 °C, pH 6.2). The degree of its inactivation during the incubation for 24 h was evaluated as 35.0% (Table IV). Therefore, a 65.0% part remained active which was still of an adequate amount to warrant a sufficient incorporation of L-methionine, since even the $[E]_0/[S]_0$ value of 0.005 (=0.5%) was found effective enough (Table IV).

Besides the enzyme amount, the concentration of L-cysteine is an important factor influencing the cost of manufacturing the product. Although most experiments were conducted at an excessive concentration of this activator, its irreducible minimum was found to be as low as 1.0 mM and the like (Table V).

The material cost of L-methionine may be another important factor in an economic point of view. Use of racemic DL-methionine, however, could solve this problem to an extent. Figure 3 shows that just the L-form fraction of the racemate can be utilized to be incorporated, the D-Met-OEt remaining intact during the incubation. Furthermore, Table VI indicates that D-Met-OEt is stable even to chemical hydrolysis into free D-methionine. The D isomer thus resolved as a result of the present one-step process could be reused by racemization.

CONCLUSION

(1) L-Methionine was efficiently incorporated into SPI by incubating it with papain in the presence of L-Met-OEt under unusual conditions.

(2) A most preferable set of the conditions was established as follows: $[S]_0 = 20$ (wt %), $[M]_0/[S]_0 = 0.05$ (w/w), $[E]_0/[S]_0 = 0.01$ (w/w), $[A]_0 = 1.0$ (mM), $pH_0 = 10.0$, $t = 8$ (h) at 37 °C.

(3) This process is characterized especially by a very high substrate concentration and an alkaline pH which is unusual for papain action.

(4) It would be interesting to elucidate the mechanism of the incorporation reaction in special relevance to such specificities of the conditions.

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Received for review July 10, 1978. Accepted August 29, 1978.

Retention of Aliphatic Alcohols by Anhydrous Lactose

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The retention of aliphatic alcohols by anhydrous α-lactose, prepared from α-lactose monohydrate by treating with the appropriate alcohol or by heating α-lactose monohydrate, has been measured by gas chromatographic and by proton magnetic resonance methods. The results are compared with those reported for retention of alcohols by adsorption from the vapor phase or by freeze-drying of aqueous alcoholic solutions. The retention decreases in the order methanol > ethanol > 1-propanol = 1-butanol. β-Lactose prepared by crystallization from aqueous solution does not take up methanol, whereas β-lactose prepared by anomerization of α-lactose in methanol or ethanol retains these alcohols at levels comparable to those found for α-lactose.

Ross (1978) reported that methanol treatment altered several physical properties of lactose, including melting point, heat of fusion, heat capacity, and density. Furthermore, the anhydrous form of α-lactose, α_M, prepared by refluxing α-lactose monohydrate in absolute methanol (Lim and Nickerson, 1973), was shown to differ from α-lactose monohydrate and from the stable, anhydrous species, α_S, produced by heating in air (Sharp, 1943). Two facts argued against the presence of adsorbed methanol in α_M: the samples had been dried to constant weight at 60 °C under vacuum, and no desorption peak was detected in the differential scanning calorimetry (DSC) thermograms, whereas even the tightly held water of crystallization desorbs from α-lactose monohydrate with a characteristic endothermic peak (Berlin et al., 1971). Nevertheless, we thought that the presence or absence of methanol in α_M should be confirmed directly. Other alcohols which are known (Nickerson and Lim, 1974) to remove the water of hydration from α-lactose monohydrate were also included in the study. We have designated the products of treating α-lactose monohydrate with ethanol, 1-propanol, and 1-butanol as α_E, α_P, and α_B, respectively.

MATERIALS AND METHODS

Chemicals. The lactose samples used in this study were prepared by treating α-lactose monohydrate (Sigma Chemical Company, St. Louis, Mo.) with various alcohols at reflux temperature for 2 h or at 27 °C for 16 h. After being cooled to room temperature, the lactose was removed by filtration, washed with the appropriate alcohol, and then dried in a vacuum oven (9 mmHg) at 60 °C until the change in weight of a 15-g sample was less than 1 mg. Constant weight was attained in 24 to 48 h provided the sample thickness did not exceed 1 cm. Lactose samples prepared from the different alcohols were dried separately in order to prevent contamination by another alcohol.

Anhydrous α-lactose, α_S, prepared from α-lactose monohydrate by heating in air in 130 °C for 3 h (Sharp, 1943), was treated similarly with the various alcohols.

Hygroscopic α-lactose, α_H, prepared from α-lactose monohydrate by heating in vacuum at 130 °C for 16 h (Herrington, 1948), and β-lactose, prepared by crystallization from boiling, aqueous solution (Buma and van der Veen, 1974), were treated with methanol. β-Lactose was also prepared by anomerization of α-lactose monohydrate with potassium methoxide in methanol or ethanol (Parrish et al., 1978).

Portions of the alcohol-treated lactose samples were heated in air at 130 °C for 16 h.

Analytical Procedures. Purity of lactose samples was determined by measuring the optical rotation of replicate solutions in water (2–5%) with a Perkin-Elmer Model 141 automatic polarimeter. Calculations were based on the latest available specific optical rotations at 589 and 546 nm (Buma and van der Veen, 1974).

Purity was also determined from the shape of the DSC fusion endotherms at 1 °C/min programming rate (Sondack, 1972). A DuPont Model 990 Thermal Analyzer was used for these determinations, as well as for measurements of melting points. Procedures for instrument

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