CHROM. 3844

Differentiation of native and renatured alkaline milk phosphatase by means of Sephadex gel thin-layer chromatography

In some cases a pasteurized milk or cream having a negative phosphatase reaction may show a weak positive phosphatase activity again after having been stored for some time. The occurrence of such a reactivation may cause serious difficulties when samples of milk or cream are tested for correct pasteurization, as it is impossible to distinguish between positive phosphatase tests caused by native and by reactivated phosphatase, when using the analytical methods at present available. The methods described in the literature are unsatisfactory. However, when using thin-layer gel chromatography on Sephadex G-200 gels in combination with agar gel electrophoresis, we found a pronounced difference between the isozyme patterns of native and of reactivated phosphatase.

Experimental

Thin-layer gel chromatography was carried out on Sephadex G-200 superfine gel having a thickness of 0.5 mm (ref. 1) and the glass plates were 20 × 40 cm. Development was with a phosphate buffer pH 7.0, descending at an angle of 15–20°. Detection of the proteins and enzymes was performed on a Whatman III MM filter paper replica of the Sephadex layer either by a normal protein-staining procedure with Lissamine Green or by detection of phosphatase activity by incubating with the substrate sodium β-naphthylphosphate and the diazo reagent Fast Red TR.

Agar gel electrophoresis was carried out according to the method of WIEME[2]. Buffer: Barbital-Na, pH 8.4, $\mu = 0.05$. Current: about 25 mA per glass slide. Detection of the enzyme zones on agar gel was by incubation with sodium β-naphthylphosphate (0.15% in borate buffer pH 9.6) and Fast Blue 2 R (0.15%). The enzyme extracts, prepared according to the procedure of MORTON[3] were concentrated by osmosis against Carbowax 20 000 in a Colover Concentrating Cell (L.K.B.).

Under-pasteurization was carried out by heating the cream for 1 min at 65–66° in a laboratory apparatus. The cream was centrifuged for 45 min at about 3000 r.p.m. at 30°; the skim milk obtained is called the serum phase. High temperature pasteurization was performed by heating the cream for two seconds at 90° in a laboratory apparatus. Reactivation of the phosphatase activity was carried out by storing high temperature pasteurized cream for 24 h at 30° after adding 1.5 ml 4.9 *M* $MgCl_2$ per 100 ml of cream.

The phosphatase activity of the serum phase of samples of under-pasteurized cream, determined according to SANDERS AND SAGER[4] is about 20–1000 μg phenol per ml; the phosphatase activity of the serum phase of reactivated cream varies from 20 up to 800 μg phenol per ml.

Polyacrylamide (disk) electrophoresis was performed in a "Pleuger" apparatus. The concentration of the gel was 7.5% of polymer. Buffer: Tris–glycine, pH 8.5.

Results and discussion

The difference between native and reactivated phosphatase was investigated by thin-layer gel chromatography on Sephadex G-200 gels and also by agar gel electro-

phoresis[5]. The enzymes are separated from cream by extraction with butanol according to MORTON[3]. This extraction causes the phosphatase enzyme to be split from lipoprotein complexes with which it is associated. By gel chromatography the native phosphatase enzyme is separated into four distinct fractions having different molecular weights; the separated protein fractions are indicated as the fragments AP_I, AP_{II}, AP_{III} and AP_{IV} (ref. 6). The molecular weight was estimated by comparison with standard compounds having well-known molecular weights and graphical interpolation of R_F values. The four fragments were quantitatively evaluated by measuring the optical density of the coloured spots with the Chromoscan densitometer (Messrs. Joyce and Loebl).

By means of agar gel electrophoresis, the alkaline phosphatase enzyme was shown to consist of three isozymes, namely α, β and γ-phosphatase[5]. An extract of native enzyme, having α- and β-phosphatase activity on agar gel, produces the four fragments AP_I, AP_{II}, AP_{III} and AP_{IV} on a Sephadex chromatogram. Enzymatic activity can only be demonstrated in the AP_{III} and AP_{IV} spots. The AP_{III} fragment has been identified as the α-AP isozyme and the AP_{IV} fragment as the β-AP isozyme[6]. The serum phase of underpasteurized cream giving a positive phosphatase test, produces the characteristic patterns of the four fragments on Sephadex, although the AP_{IV} spot present here is rather small (about 10%, see Table I). On Sephadex,

TABLE I

COMPOSITION OF ALKALINE PHOSPHATASES AND PROTEIN FRAGMENTS WITH DENSITOMETRIC EVALUATION OF SEPHADEX G 200 "CHROMATOGRAMS"

Fragment of alkaline phosphatase	*Serum phase under-pasteurized cream*	*Serum phase pasteurized cream*	*Serum phase reactivated cream (β_2)*	*Serum phase reactivated cream (β_3)*	*Mol. weight by Sephadex chromatography*	*Mol. weight average*
AP_I	25%	25%	40%	~40–70%	15,000–22,000	18,000
AP_I'	0	25%	0	0	33,000–39,000	36,000
AP_{II}	35%	0	0	~10–15%	45,000–65,000	55,000
AP_{III} (α-AP)	30%	40%	0	0	120,000–160,000	140,000
AP_{IV} (β-AP)	10%	10%	60%	~10–45%	450,000–650,000	570,000

an enzyme extract prepared from the serum phase of pasteurized cream (in which therefore only denatured, inactive phosphatase is present) produces no AP_{II} fragment, but an AP_I' spot corresponding to a somewhat different molecular weight.

On agar gel, the enzyme pattern of pasteurized, reactivated cream apparently may vary considerably[6]. In some samples of cream, the activity of the β_1 or the β_2-enzyme, exclusively, is demonstrated, in other samples both the β_1 and β_2-enzyme are found and in special cases a faster moving spot, the so-called β_3-AP enzyme, appears between the spots of α and β_2-phosphatase.

In the *serum phase* of raw or under-pasteurized cream, the α-phosphatase enzyme is found exclusively, but in the serum phase of reactivated cream we have demonstrated the β_2 and β_3-AP isozymes. This characteristic difference may be used for distinguishing between native and renatured AP isozymes.

In the serum phase of reactivated cream (β_2-phosphatase activity on agar gel)

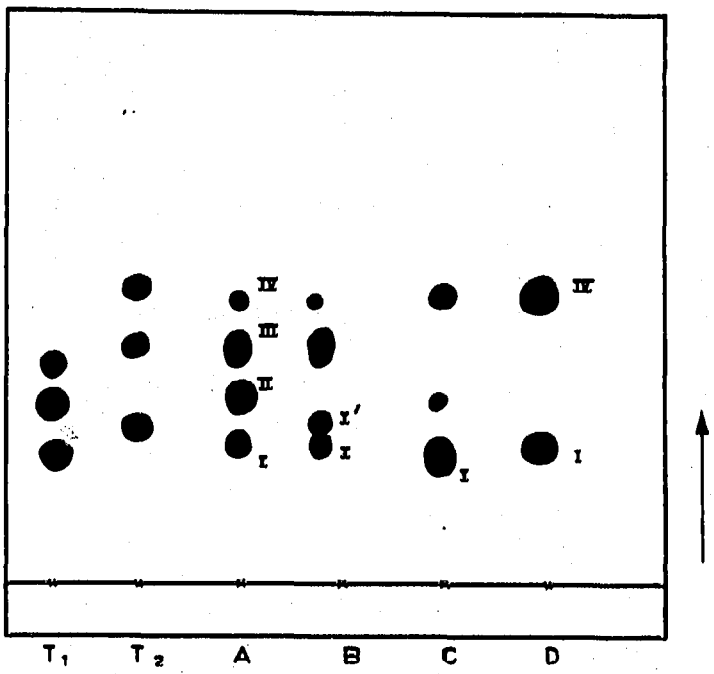

Fig. 1. Separation of alkaline phosphatases by permeation chromatography on Sephadex G 200 (superfine). Development (descending) with phosphate buffer pH = 7.0; dimensions of glass plate: 20 × 40 cm. Test proteins with known molecular weight: T_1 = Lysozyme, β-lactoglobulin and serum albumin (bovine); T_2 = α-chymotrypsinogen-A, γ-globulin (human) and thyroglobulin (mol. wt. 670,000). (A) = Phosphatase isozymes from the centrifuged aqueous phase (skim milk) of under-pasteurized cream; protein staining with Lissamine Green; (B) = phosphatase isozymes from the serum phase of pasteurized cream, obtained from a dairy; (C) = phosphatase isozymes from the serum phase of reactivated cream having β_3-activity; (D) = phosphatase isozymes from the serum phase of reactivated cream having β_3-activity.

only the fragments AP_I and AP_{IV} are found on Sephadex. The intensity of the AP_{IV} spot is very pronounced here (about 40–70%). In the serum phase of reactivated cream having β_3-enzyme activity (on agar gel), a clearly different pattern is observed on Sephadex. Phenomena which are remarkable are the presence of a strong AP_I fragment having a molecular weight which is generally lower than the normal value of AP_I and, in addition, the absence of the AP_{III} fragment (see Fig. 1). Therefore the β_3-isozyme on agar gel may also be characterized by the pattern of AP_I and AP_{IV} fragments on Sephadex gels. If a strong AP_I fragment is present in the serum phase of reactivated cream, the intensity of the AP_{IV} fragment is generally rather low (about 10–20%). However, with some samples of reactivated cream a rather strong AP_{IV} spot is observed in the serum phase (about 25–40%). The fat phase of reactivated cream also shows a strong AP_I fragment having a low molecular weight of about 14 000 and, naturally, also a very strong AP_{IV} zone.

Therefore a characteristic difference is that the AP_I fragment of reactivated phosphatase enzyme has a strong intensity, whereas this fragment is present in relatively slight amounts in the native enzyme.

A mixture of under-pasteurized and reactivated cream can be analysed very well by means of gel chromatography on Sephadex G-200 gels and agar gel electrophoresis. In the serum phase of this mixture a strong AP_{IV} fragment is found on Sephadex, which indicates the presence of reactivated phosphatase. With agar gel

electrophoresis, exclusively α-AP isozyme is observed on the pherogram, from which the presence of native phosphatase can be inferred.

With the intention of investigating further the behaviour of the AP_I spot with low molecular weight, the serum phases of under-pasteurized and reactivated cream (β_3-AP) were analysed on Sephadex G75. A marked separation between the fragment AP_I and the fragment having a lower molecular weight was observed. The latter may provisionally be indicated by AP_0. However, it cannot be excluded that this AP_0 fragment may be an artefact formed during preparation and especially during concentration of the enzyme extract. With agar gel electrophoresis the presence of the β_2- or β_3-isozyme in the serum of a reactivated cream already points to a significant difference in behaviour between the serum phases of under-pasteurized and reactivated cream, as in the serum of raw or under-pasteurized cream on agar gel the α-phosphatase exclusively is always found. From the experiments mentioned it therefore appears that both by gel chromatography and by agar gel electrophoresis a characteristic difference may be observed between native and reactivated phosphatase enzyme (see Table I).

The molecular weights show that the AP_I fragment may be a (perhaps "elementary"?) subunit of the phosphatase enzyme; this subunit, whose molecular weight is about 18 000, is present in the majority of the extracts as a kind of degradation or dissociation product[6]. The AP_I' fragment, present in pasteurized cream, has a molecular weight of about 36 000 and represents a degradation product that is probably composed of two subunits having a molecular weight of 18 000. The isozymes α-AP (fragment AP_{III}) and β-AP (fragment AP_{IV}) are possibly composed of a number of such subunits having a molecular weight of 18 000 (see Table II).

From our experiments it is therefore apparent that especially the *serum phase* of cream or milk should be analysed in order to demonstrate the pronounced difference between native and renatured phosphatase enzyme.

A characteristic difference in the protein patterns of the serums of raw and reactivated cream is also apparent when using polyacrylamide gel electrophoresis. Proteins having a low molecular weight generally show the greatest mobility on polyacrylamide gels, which is caused at least partially by a permeation effect, whereas proteins having a very high molecular weight remain practically at the top of the column. This difference is illustrated in Fig. 2. Zone III is found in native phosphatase, but is practically absent in renatured phosphatase. Therefore we may sup-

TABLE II

FRACTIONATION OF ALKALINE PHOSPHATASES AND PROTEIN FRAGMENTS ON LAYERS OF SEPHADEX G 200 WITH AN APPROXIMATE ESTIMATION OF THEIR MOLECULAR WEIGHTS

Fragment	*Mol. wt.*	*Multiplication factor**	*Identification of spot*
AP_I	18,000	1	elementary subunit
AP_I'	36,000	2	degradation product
AP_{II}	55,000	~4	degradation product
AP_{III}	140,000	~8	α-AP
AP_{IV}	570,000	~32	β-AP

* With respect to mol. wt. = 18 000 of subunit.

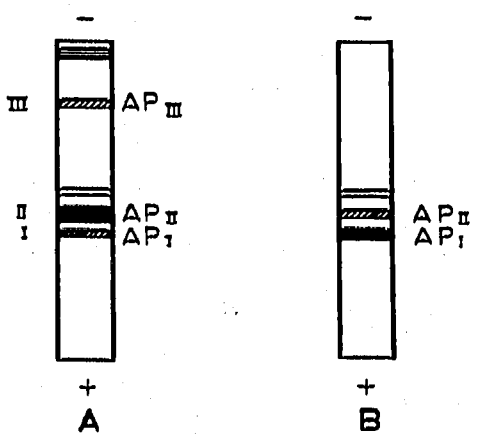

Fig. 2. Separation of alkaline milk phosphatases by disk electrophoresis in gels of polyacrylamide (7.5%). Buffer: tris–glycine pH 8.5; staining with Amido Black 10B (1%) in 7% acetic acid solution. (A) = Phosphatase isozymes from the centrifuged aqueous phase of underpasteurized cream; (B) = phosphatase isozymes from the serum phase of reactivated cream.

pose that this zone III is identical with the AP_{III} fragment on Sephadex (that means with the α-AP isozyme), as the AP_{III} fragment is absent in reactivated phosphatase as well when applying Sephadex gel chromatography. In the same manner zone II may probably be identified with the AP_{II} fragment. In raw cream, this protein is always present on polyacrylamide gel as a strongly coloured zone, which therefore agrees with the results obtained by chromatographing on Sephadex gel, as in the latter case the AP_{II} fragment likewise is seen as a rather intensely coloured spot (about 35%; see Table I). On the other hand, the serum of reactivated cream only shows a weak zone II on polyacrylamide gels, and on Sephadex gels the AP_{II} fragment is also observed at a lower concentration of about 15%.

The protein zone I, that is the zone having the greatest mobility, shows resemblance to the AP_I fragment which has a molecular weight of about 18 000. With Sephadex gel chromatography in most cases a relatively high concentration of AP_I fragment (about 40–70%) is found in the serum of reactivated cream. On polyacrylamide gels zone I is relatively pronounced, whereas the serum of raw cream only produces a faint zone I. The protein patterns of the enzyme extracts isolated from the serum of raw cream and of reactivated cream therefore show characteristic differences, which likewise may be used for distinguishing native and renatured phosphatase isozymes.

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