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Immune Competence-Promoting Action of Bovine Parotid Hypocalcemic Factor obtained *via* Extraction with Glacial Acetic Acid[1]

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The hypocalcemic substance P–MSY (M.W. 66000) and a few partial purified products obtained through the glacial acetic acid extraction of bovine parotid extract were examined for immune competence. Lymphocytes/polymorphs ratio increasing action was examined using the littermates of Swiss-Webster strain mice, and plaque-forming cells increasing action and rosette-forming cells increasing action were done using those of ICR strain mice.

By the dosage of 0.1 μg/mouse, the sample P–MSY produced L/P ratio of 2.72±0.12, and the effect was significant at 1% level of significance against the control group. PFC and RFC activities were significant at below 5% level of significance against the respective control group in a dose of 2.5 μg/mouse (PFC/10^6 cells: 53.57±14.57, RFC/10^6 cells: 13854±2156). From these results, L/P activity of P–MSY was increased by about 2000 times that of acetone-dried powder PAI, starting materials, and the increases in PFC and RFC activities were both about 80 times. Through the assay of P–MSY and partial purified products, a tendency to increase in L/P, PFC, and RFC activities was found in proportion of hypocalcemic activity. The regression of L/P and PFC activities against log doses were examined, and it was concluded that each response revolved to a line over a moderate range of doses. Meanwhile, P–MSY was compared with the series of other products from thymus.

Keywords——hypocalcemic protein; purified Parotin; parotid gland; immune competence; lymphocyte-increasing action; plaque-forming cell (PFC); rosette-forming cell (RFC); newborn littermate

Previously, the hypocalcemic protein (purified Parotin) with molecular weight of 48000, was isolated[3] by purification of the fraction precipitating at pH 5.4 (crude Parotin) from the aqueous extract of bovine parotid gland, and it was found to promote the immune competence.[4] Recently, we have purified a hypocalcemic protein (P–MSY) from the fraction obtained through the glacial acetic acid extraction of a fraction precipitated by addition of acetone to the supernatant left after separation of the crude Parotin from the aqueous extract of the gland. Its molecular weight (66000) and amino acid composition have been reported.[5]

1) Presented at the 97th Annual Meeting of Pharmaceutical Society of Japan, Tokyo, April 1977.
2) Location; *Tanabe-dori, Mizuho-ku, Nagoya, 467, Japan.*
3) A. Mizutani, T. Kitamura, N. Yamada, H. Inaba, Y. Naito, and T. Mizutani, *Chem. Pharm. Bull.* (Tokyo), **22**, 1955 (1974).
4) A. Mizutani, T. Mizutani, I. Suzuki, and P–F. Kuo, *Chem. Pharm. Bull.* (Tokyo), **25**, 1601 (1977).
5) I. Suzuki, H. Yamamoto, and A. Mizutani, *Seikagaku* (in Japanese), **48**, 466 (1976).

The present paper describes the effects of P-MSY and of a few partial purified fractions obtained in the process of purification on lymphocyte-increasing action (L/P activity), plaque-forming cells increasing action (PFC activity), and rosette-forming cells increasing action (RFC activity) in comparison with those activities of the purified Parotin above mentioned.

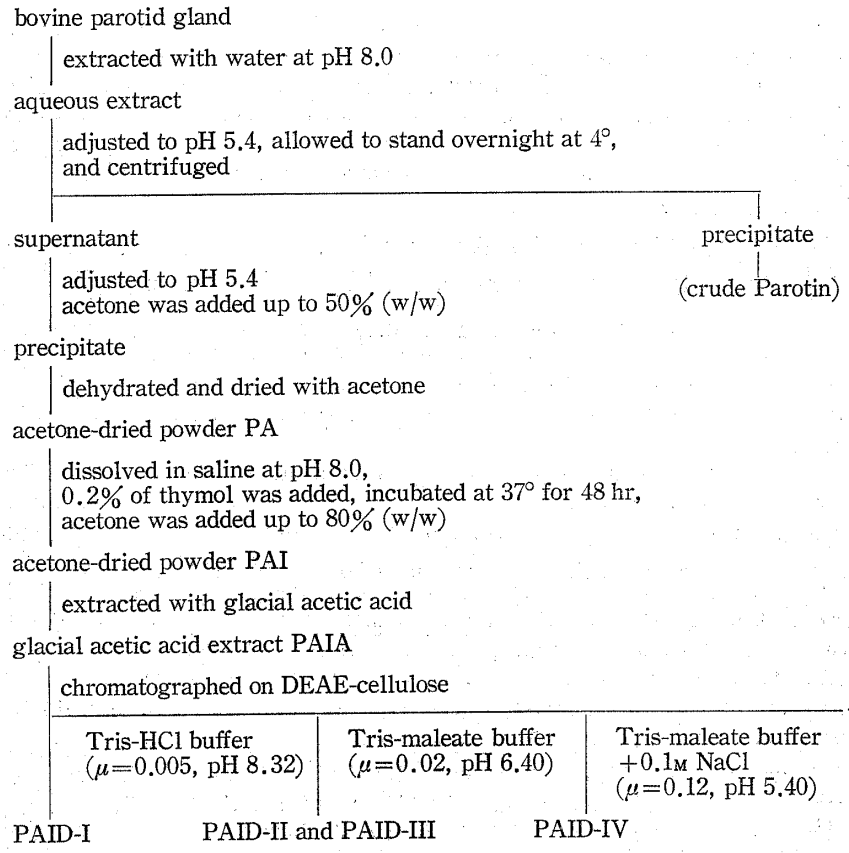

Fig. 1. Hypocalcemic Fractions obtained from Bovine Parotid Extract

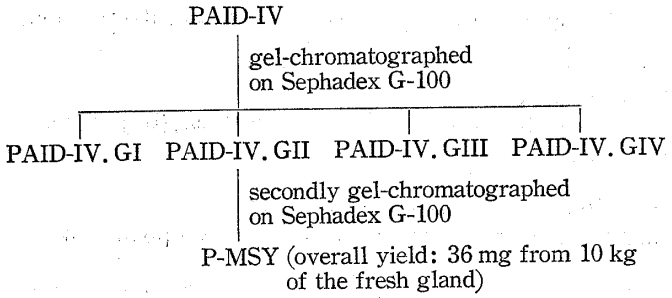

Fig. 2a. Purification of the Hypocalcemic Substance by Gel Chromatography on Sephadex G-100

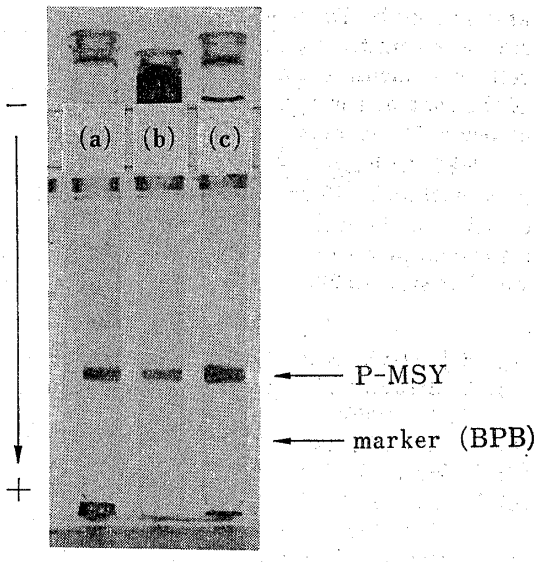

Fig. 2b. Disc Electrophoresis (7.5% gel, pH 8.6) of PAID-IV obtained by DEAE-cellulose Chromatography (a), and PAID-IV. GII obtained by First Gel Chromatography (b), and P-MSY purified by Second Gel Chromatography on Sephadex G-100 (c)

Materials and Methods

Materials——The sample P-MSY and the products obtained in the course of purification were prepared by the previously reported method,[5] and the process is outlined in Figs. 1 and 2. Acetone-dried powder, PA, obtained from the supernatant after separation of crude Parotin was suspended in saline around at pH 8.0, and through the incubation of the suspension in the presence of 0.2% of thymol, the second acetone-dried powder, PAI, was prepared. Fraction PAI was extracted with glacial acetic acid and the lyophilized product, PAIA, was obtained. Fraction PAIA was fractionated by DEAE-cellulose chromatography, and fraction PAID-IV was obtained (Fig. 1). The product P-MSY giving single band in disc electrophoresis (7.5% gel, pH 8.6)[6] (Fig. 2b) and electrofocusing, was purified from twice repeated gel chromatography of PAID-IV on Sephadex G-100 (Fig. 2a). The yield of fraction P-MSY was about 36 mg from 10 kg of the fresh gland. Ovalbumin (Wako Pure Chemical Industrial Ltd.) was used as a control.

Measurements of Biological Activities

Hypocalcemic Activity——Hypocalcemic activity was assayed by the method described previously.[7] A group of 6 mature male rabbits was injected with sample dissolved in saline in a dose of 0.5 ml/kg. Blood was drawn before the injection, and 4, 5, and 6 hr after the injection. Out of three serum samples taken after the injection, the value of maximum lowering in serum calcium was determined, and its percent decrease was calculated against the calcium value before the injection, then averaged for the 6 animals. The difference between this mean value and the mean value obtained from the control 6 animals injected with saline was examined by the *t*-test, and the value giving a significant difference at below 5% level of significance was taken as being effective.

L/P Activity——Metcalf's method[8] was employed with a little modification.[9] The experimental animals were newborn Swiss-Webster strain mice of 6—12 hr after birth. The litter was divided into two groups; one group was given a sample and the other saline as a control, both by intraperitoneal injection. Blood was drawn from the tail before the injection, and 6, 10, and 14 days after the injection. Differential white cell counts were made using Wright's stain,[10] and lymphocytes/polymorphs (L/P ratio) was calculated. The mean of the increment of L/P ratio against L/P ratio before the injection was calculated for each of blood samples obtained from the control and test groups, and the difference in the mean between the test and control groups was examined by the *t*-test on each day bled according to the method described in our previous paper.[11] If one of these blood samples showed a significant difference at less than 5% probability the sample tested was considered to be effective.[12]

PFC Activity——The modified method of Jerne and Nordin[13,14] was employed. The littermates of neonatal mice of ICR strain within 6—12 hr after birth were divided into two groups. In the same manner as the case of L/P activity, one group was injected with a sample solution and the other with saline. After 14 days, the mice of both groups were injected with 20% sheep red blood cells (SRBC), and spleen was excised 4 days later. Spleen cell suspension was prepared by lossened the spleen into small pieces in 0.9% Eagle's MEM solution and it was used for the development of PFC on an agar plate through almost the same procedure as described by Doresser and Greaves.[15] Meanwhile, the number of cells in the above mentioned cell suspension was counted by using a melangeur and a Bürker-Türk hemocytometer. The number of PFC per 10^6 cells was calculated, and the *t*-test was made on the difference between the mean of the test group and that of the control group, and the sample tested was considered to be effective when the difference was significant at below 5% level of significance.

RFC Activity——The method of Dresser and Greaves[15] was employed. The littermates of ICR strain mice within 6—12 hr after birth were used. The injection of the test sample and SRBC, and subsequent excision of the spleen were performed in the same manner as in the case of PFC. The spleen was loosened into small pieces in 2 ml of Dulbecco's phosphate buffer in the absence of Mg^{2+} and Ca^{2+}, and strained with a stainless sieve of 200 mesh, then the cells were washed with the same buffer solution 3 times by centrifugation.

6) B.J. Davis, *Ann. N. Y. Acad. Sci.*, **121**, 404 (1964).
7) A. Mizutani, M. Terada, Y. Toda, and K. Yamamoto, *Ann. Pept. Pharm. Nagoya City Univ.*, (in Japanese) **17**, 16 (1969).
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9) T. Hand, P. Caster, and T.D. Luckey, *Biochem. Biophs. Res. Commun.*, **26**, 18 (1967).
10) S. Hino, (ed.), "Textbook on Clinical Test Techniques," (in Japanese) Vol. 15, Ishiyaku Shuppan, Tokyo, 1972, p. 143.
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15) D.W. Doresser and M.F. Greaves, "Handbook of Experimental Immunology: Cellular Immunology," Second Edition Vol. 2, ed. by D.M. Weir, Blackwell Scientific Publications, London, 1973, p. 27.1.

To the sedimented cells, 2 ml of 0.9% Eagle's MEM solution was added to give cell suspension. The aliquots of 0.4 ml of the cell suspension were mixed with 0.2 ml of 2% SRBC and centrifuged at 1000 rpm for 6 min at 4°, followed by gentle stirring. The resulting suspension was placed in a Bürker-Türk hemocytometer, and the rosettes formed were counted using dark-field phase contrast microscope. The number of RFC per 10^6 cells was determined, and the mean values were calculated for the test and control groups. The RFC effect was examined by the *t*-test as in the case of PFC.

Results and Discussion

L/P and PFC activities are shown in Table I, including hypocalcemic activity which is reproduced from our previous report.[5] The upper and lower rows give the mean values of

Table I. Hypocalcemic, L/P, and PFC Activities of the Fractions obtained from Parotid Gland Extract

Materials injected	Hypocalcemic activity (Rabbits)		L/P activity (Swiss-Webster mice)			PFC activity (ICR mice)		
	Dose (mg/kg)	Percent decrease in serum Ca Mean±S.E. (n=6)	No. of animals (n)	Dose (μg/mouse)	Ratio Mean±S.E. Upper: Sample Lower: Control	No. of animals (n)	Dose (μg/mouse)	No. of PFC/10^6 cells Mean±S.E. Upper: Sample Lower: Control
PAI	10	15.82±4.07[a]	6	200	1.72±0.07[b,c]	5	200	71.90±25.21[a]
			5		1.18±0.03	5		7.93± 2.97
PAIA	0.1	13.43±1.14[b]	5	10	1.86±0.06[a,d]	7	80	66.82±16.84[a]
			5		1.32±0.10	5		10.83± 2.46
PAID-IV	0.05	10.12±0.74[b]	6	2	2.95±0.22[b,d]	4	40	71.46±12.43[b]
			6		2.04±0.14	4		6.60± 2.03
P-MSY	0.01	10.67±1.65[b]	4	0.5	3.26±0.25[b,d]	7	5	93.34±27.85[a]
			3		1.65±0.13	6		17.64± 4.22
	0.005	7.90±1.05[a] (×2000)	4	0.25	2.95±0.29[b,d]	4	2.5	53.57±14.57[a]
			4		1.66±0.07	4		15.34± 3.65 (×80)
	0.0025	5.83±1.25	5	0.1	2.72±0.12[b,d]	5	1	18.73± 5.96
			5		1.82±0.07 (×2000)	4		10.08± 2.91
Ovalbumin 4		4.81±1.16	5	20	1.22±0.03[c]	5	20	15.98± 6.95
			4		1.18±0.06	4		18.20±12.73
Nonimmunized		—			—	5		1.90± 0.72

a) Significantly different from control, $p<0.05$.
b) Significantly different from control, $p<0.01$.
c) The data were obtained at 10 days after birth.
d) The data were obtained at 14 days after birth.
The x number parenthesized represents rouahly the number of times by which the potency of sample rose over that of acetone-dried powder PAI.

samples and controls, respectively. In proceeding the successive purifications from PAI to PAIA, PAID-IV, and P-MSY, both PFC and L/P activities were increased relating to the increase in hypocalcemic activity. In the case of L/P activity, PAI was effective in a dose of 200 μg/mouse, while P-MSY was effective in a dose of 0.1 μg/mouse, hence the activity was increased by about 2000 times that of PAI, and hypocalcemic activity was also increased by about 2000 times. Fraction PAI had the significant PFC activity in a dose of 200 μg/mouse, whereas P-MSY had the significant activity in a dose of 2.5 μg/mouse, and the increase in the activity was about 80 times. Ovalbumin did not give any significant effect on PFC activity and L/P activity at 20 μg/mouse, and hypocalcemic activity at 4 mg/kg. In case of the non-immunized mice into which SRBC was not injected, PFC/10^6 cells resulted in a very small value of 1.90±0.72. This value coincides well with that reported by Friedman (2—3

PFC/10^6 cells).[16] RFC activity was increased as proceeding of the purification like PFC activity, as shown in Table II. Fraction PAI was effective in a dose of 200 μg/mouse, while

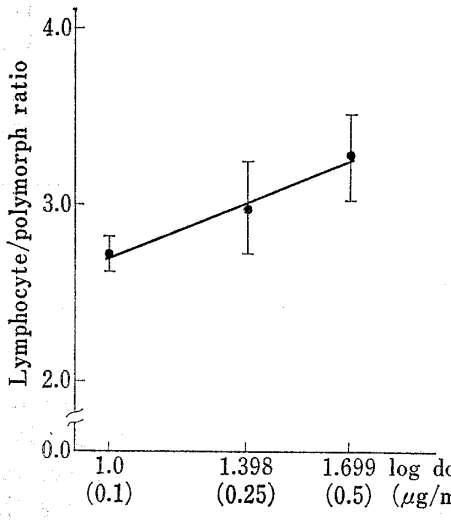
Table II. RFC Activity of the Fractions obtained from Bovine Parotid Gland Extract

Materials injected	Dose (μg/mouse)	No. of animals (n)	Body weight (g)	Spleen weight Upper: Sample Lower: Control (mg)	(%)	No. of RFC/10^6 cells Upper: Sample Lower: Control Mean±S.E.
PAI	200	5	8.2	67.8	1.5	19541±1394[a]
		5	8.6	66.8		6825±1413
PAIA	80	4	10.8	95.7	26.6	15208±1281[a]
		4	10.3	75.6		7918± 715
P-MSY	2.5	4	7.8	87.1	32.2	13854±2156[b]
		4	7.5	65.9		7519± 974
Nonimmunized	—	5	13.4	94.5		1624± 233

a) Significantly different from control, $p<0.01$.
b) Significantly different from control, $p<0.05$.

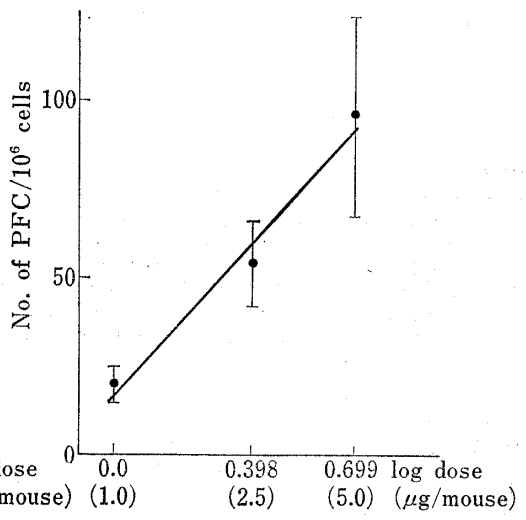
P–MSY gave the mean value of RFC/10^6 cells as to be 13854±2156 in a dose of 2.5 μg/mouse, and was significant at 5% level of significance. Thus, the activity of P–MSY was increased by about 80 times that of PAI as in the case of PFC activity. The non-immunized mice produced large value of 1624±233 RFC. The values agrees well with that obtained Laskov (100—1000 RFC/10^6 cells),[17] and was extraordinarily larger than PFC value. However, the potency of the material for RFC activity was almost the same as that for PFC activity because the value of the control group was also large in the assay for RFC activity.

Hand *et al.*[18] reported the remarkable increase in the weight of spleen of a mouse received lymphocyte-stimulating hormone (LSH). On the other hand, in our present work the glacial acetic acid extracts and its purified products showed a little increase in weight (20—30%, Table II). In the PFC method, it seems difficult to detect IgG antibody because of



$Y=0.75X+3.45$

Fig. 3a. Dose-response Curve of P-MSY for L/P Activity



$Y=106.85X+16.80$

Fig. 3b. Dose-response Curve of P-MSY for PFC Activity

16) H. Friedman, *Proc. Soc. Exptl. Biol. Med.*, **117**, 526 (1964).
17) R. Laskov, *Nature*, **219**, 973 (1968).
18) T.D. Luckey, W.G. Robey, and B.J. Campbell, "Thymic Hormones," ed. by T.D. Luckey, Univ. Park Press, Baltimore, 1973, p. 175.

its low hemolytic competence,[19] while in the RFC method the sensitivity against IgG antibody is reported to be high.[15] Meanwhile, RFC method is based on detection of cells to which antibody was adhered, and involved some risk of counting the cells not forming antibody.

In the analysis of variance on the regression of L/P ratio to log doses of 0.1, 0.25, and 0.5 μg/mouse using P-MSY, the linearity of dose-response relationship was recognized at 1% level of significance and it reduced to the regression line given by an equation of $Y=0.75X+3.45$ (Fig. 3a). In the case of PFC activity, the linearity was also recognized on PFC/10^6 cells to log doses of 1.0, 2.5, and 5.0 μg/mouse with 1% significance and the regression line was $Y=106.85X+16.80$ (Fig. 3b). Consequently, both L/P and PFC activities showed the fair linearity over the moderate range of doses. These results suggest the availability of this method for more accurate assay on immune response. It is considered to be a main reason for obtaining good results, that we used, for the experiments, two groups (test and control) of mice obtained from newborn littermates.

The substance P-MSY was found to have 4—5 times stronger the promotion of immune competence than the purified Parotin, since the latter showed effective PFC activity in a dose of 10 μg/mouse, and L/P activity in a dose of 0.5 μg/mouse.[11] In comparison with two hypocalcemic protein (TP_1 and TP_2) isolated from bovine thymus by Mizutani *et al.*, P-MSY showed almost the same immunological activity as that of TP_1 and TP_2 since they have the effective PFC activity in a dose of 1 μg/mouse and L/P activity in a dose of 0.1—0.2 μg/mouse.[12,20] Robey *et al.*[21] reported that LSHr isolated from bovine thymus extracts showed effective L/P activity in a dose of 8 μg/mouse and PFC activity in a dose of 0.1 μg/mouse in 5 of 9 mice. However, a definite comparison could not be made because of the different assay method.

On the bases of these facts, the substance P-MSY was found to have the strong immune competence promoting action.

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20) A. Mizutani, I. Suzuki, Y. Matsushita, and K. Hayakawa, *Chem. Pharm. Bull.* (Tokyo), **25**, 2156 (1977).
21) G. Robey, B.J. Campbell, and T.D. Luckey, *Infect. Immunity*, **6**, 682 (1972).